Characterisation of Tau splicing factors in Alzheimer's disease

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Characterisation of Tau splicing factors in Alzheimer's disease

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By

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DECLARATION

I hereby declare that with the exception of the RT-PCR analysis of tau exon 10 splicing in human brain in Chapter 3, all of the work presented in thesis is my own.

Aderonke Adelodun

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ABSTRACT

Neurofibrillary tangles consist of hyperphosphorylated tau, one of the pathological hallmarks of Alzheimer's disease (AD). Alternative splicing of the tau pre-mRNA produces six tau isoforms with or without exon 10 (E10); tau4R or tau3R, microtubule binding repeats respectively. In normal human brains the ratio of 4R/3R tau is approximately one. Aberrant E10 splicing is also observed in some sporadic AD cases as well as Pick's disease.

The rationale for this study is that abnormal splicing may predispose to neurological diseases such as AD either through abnormal expression and/or activity of splicing factors that control tau alternative splicing. In particular, expression levels of splicing factors regulating tau pre-mRNA splicing may be altered in AD and contribute to pathogenesis.

The AD model used was dependent on the presence or absence of TDP43 inclusions because approximately 30 % of AD cases have inclusions of the RNA binding protein, TDP-43 which may contribute to the clinical phenotypes observed in these cases. However TDP-43 is an RNA binding protein known to regulate alternative splicing. In addition, TDP-43 pathology has now been shown to co-occur with tau pathology in some tauopathies. Whether the TDP-43 inclusions in AD is incidental or whether it contributes to more severe clinical phenotype remains unresolved. This model was used to determine if TDP43 pathology in AD exacerbates the changes in splicing factor expression in AD cases with TDP43 pathology.

Splicing factors including serine and arginine (SR) rich proteins that either repress or promote E10 inclusion and CELF proteins are of particular interest. The brain selective CELF3 promotes tau E10 inclusion *in vitro* by binding to an intronic element in tau pre-mRNA. The levels of expression of CELF proteins and SR proteins that modulate tau splicing were characterised in five brain regions (frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum) using

post-mortem brain tissue from AD patients. Tau E10 alternative splicing pattern was also analysed in these brain regions.

We investigated the expression levels of CELF proteins (CELF1, CELF3 and CELF4) and SR proteins (SC35, SRp40, and SRp55) in AD cases with (+) and without (-) TDP-43 inclusions compared to aged-matched controls.

Tau E10 alternative splicing pattern analysis revealed that tau4R was increased in the amygdala and hippocampus of AD brain. We found, by quantitative RT-PCR, that the expression level of CELF3 RNA is increased in the amygdala and frontal cortex of AD cases, regardless of TDP-43 inclusions. Interestingly, in the amygdala of ADTDP43- cases where an increase in tau4R is found, we also found an increased expression of CELF4, SRp40 and SRp55 RNA. Although CELF3, CELF4 and SRp40 promote tau E10 inclusion *in vitro*, SRp55 inhibits tau E10 inclusion *in vitro*; whether this occurs *in vivo* is unknown. The association of abnormal expression of SR proteins and CELF proteins as well as aberrant tau E10 splicing in brain regions affected during AD pathogenesis may be a contributing factor to disease.

Independent of the role of SR proteins and CELF proteins in sporadic AD, these tau splicing factors may be therapeutic targets to correct aberrant tau splicing in tauopathies. Future work will determine whether these splicing factors (CELF3, CELF4, SRp40 and SRp55) show abnormal expression in other neurodegenerative diseases.

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ABBREVIATIONS

3R	Tau isoforms containing three microtubule-binding domains
4R	Tau isoforms containing four microtubule-binding domains
ACE	A/C rich enhancer
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
bp	Base pair
BP/A	Branch point
CBD	Corticobasal degeneration
cDNA	Complementary DNA
CELF	CUG-BP and ETR-3 like factors
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CLK	cdc2-like kinases
CNS	Central nervous system
cTNT	Cardiac troponin T
CUG-BP	CUG binding protein
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-cytidine 5'-triphosphate
dGTP	2'-guanosine 5'-triphosphate
DM	Myotonic dystrophy

DNase	Deoxyribonuclease
dNTP	2’-deoxynucleotides 5’-triphosphate
DTT	Dithiothreitol
dTTP	2’-deoxytymidine 5’-triphosphate
E10	Tau exon 10
ELAV	Embryonic lethal abnormal vision
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
FALS	Familial ALS
FBS	Foetal bovine serum
FTDP-17	Frontotemporal dementia with Parkinsonism linked to chromosome 17
FTLD-U	Frontotemporal lobar degeneration with ubiquitin positive inclusions
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK-3	Glycogen synthase kinase 3
HBSS	Hank’s balanced salt solution
hnRNP	Heterogeneous ribonucleoproteins
ICC	Immunocytochemistry
IR	Insulin receptor
ISE	Intronic splicing enhancer
ISM	Intronic splicing modulator
ISS	Intronic splicing silencer
kb	Kilobases
kDa	Kilodaltons

MAP	Microtubule-associated protein
MAPT	Microtubule-associated protein tau gene
MBNL	Muscleblind-like proteins
mRNA	Messenger RNA
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NLS	Nuclear localisation signal
OD_{260}	Absorbance at 260nM
OD_{280}	Absorbance at 280nM
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHF	Paired helical filaments
PiD	Pick's disease
PNS	Peripheral nervous system
PolyQ	Polyglutamine tract
PolyY	Polypyrimidine tract
PPE	Polypurine enhancer
Pre-mRNA	Pre-messenger RNA
PSP	Progressive supranuclear palsy
PTB	Polypyrimidine tract-binding protein
R	Purine
r.p.m.	Revolutions per minute
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein

RRM	RNA recognition motif
RS	Serine- and arginine-rich domain
RT	Reverse transcription
SALS	Sporadic ALS
SB	Sample buffer
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
snRNAs	Small nuclear ribonucleic acids
snRNPs	Small nuclear ribonucleoprotein particles
SR proteins	Serine- and arginine-rich proteins
ss	Splice site
TAE	Tris-acetate-EDTA
TAR	Trans-active response
TDP-43	TAR DNA-binding protein 43
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNRC4	Trinucleotide repeat containing 4
Tra2β	Transformer 2 β
UTR	Untranslated region
v/v	Volume/volume
w/v	Weight/volume
Y	Pyrimidine

PUBLICATIONS ARISING FROM THIS THESIS

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Chapter One

1 Tau splicing factors in neurodegenerative diseases

1.1 Introduction

Neurodegenerative diseases include a diverse range of neurological disorders characterised by the progressive loss of neuron structure and function, eventually leading to neuronal death. This occurs usually within areas of the cerebral cortex, basal ganglia, cerebellum, brain stem, motor systems and spinal cord; eventually leading to the progressive loss of motor, sensory and perceptual functions.

Neurodegenerative diseases are characterised by both cognitive and functional impairments as well as behavioural symptoms. There are several well established features of neurodegenerative diseases and Alzheimer's dementia (AD) in particular. These include inflammation; neurotoxicity caused by chemicals or other toxic events; increase in free radical species and oxidative damage; loss of nerve trophic factors (important in nerve physiology) and loss of nerve cell transmission; as well as altered metal ion homeostasis (Dyrks et al., 1993; Lovell et al., 1998). Most neurodegenerative diseases are associated with the presence of abnormal protein deposits. These protein aggregates can be in the form of extracellular deposits such as amyloid plaques or intracellular inclusions within the cell body and axons/dendrites of neurons such as tau filaments (Braak and Braak, 1992). How the chemistry of the afore-mentioned features that result in the generation of radical species contributes to the production of protein deposits and hallmarks of neurodegenerative diseases is yet to be fully understood. Moreover, understanding the molecular events that lead to dysfunction of usually 'functional' proteins is paramount to finding a cure to these diseases.

Neurodegenerative diseases are considered as multifactorial conditions, some of which have well defined genetic factors as well as several possible environmental variables. A known cause of AD, is genetic mutations linked to early onset AD including the gene for amyloid precursor protein on chromosome 21 (Kang et al.,

1987), the gene for the presenilin 1 protein on chromosome 14 (Schellenberg et al., 1992) and the gene for the presenilin 2 protein on chromosome 1 (Levy-Lahad et al., 1995); all of which modify production of amyloid-beta peptide. However, the majority of AD cases are sporadic with clinical symptoms appearing mostly after the age of 65 while 5 - 10 % are familial AD (Blennow et al., 2006). Familial AD is rare and restricted to some families around the world with its prevalence being 0.1 % of the world population (Janssen et al., 2003). Hence, the prevalence of sporadic AD implies the important role that environmental (such as toxins, viruses and head trauma/ injury) and lifestyle factors play in the development of AD. In addition, the relative importance of each environmental factor may differ between patients for different neurodegenerative diseases. Better understanding and elucidation of these factors will provide preventive measures as well as effective pharmacological therapy.

In addition, epidemiological studies have highlighted other provisional links to AD such as reduced brain size, reduced mental and physical activity during late life as well as limited educational and occupational attainment. AD is now one of the leading causes of death in developed countries in addition to cancer, stroke, cardiovascular diseases, obesity and diabetes (Blennow et al., 2006).

Another prominent risk for development of AD is the presence of apolipoprotein E ε4 allele (*APOE* ε4) which increases susceptibility for AD (Corder et al., 1993; Poirier et al., 1993). Specifically, people with a first-degree relative with AD have 10 - 30 % increased risk of developing the disorder (van Duijn et al., 1991). Furthermore, the presence of ApoE in neuritic plaques led to investigation of its role in plaque formation. ApoE has been implicated in the transport of cholesterol in the brain and recycling of other lipids. Also, ApoE is involved in neuronal repair and regeneration of dysfunctional connections between neurons (Poirier, 1994). Hence, it is conceivable that ApoE production is increased in areas of the brain with Aβ as a result of neuronal damage. However, ApoE binds to Aβ (generated for APP breakdown) readily and so ApoE bound to beta amyloid is not free to repair neurons, creating the environment for plaque formation (Carter,

2005; Holtzman et al., 2000). Therefore, the binding of ApoE to beta amyloid assists paque formation.

Recently, the genome-wide association studies (GWAS) in AD have highlighted over a dozen novel genetic risk factors associated in AD. These include:

- *GAB2* encodes GRB2-associated binding protein 2 (Gab2) a scaffolding protein invoveld in signaling and transduction pathways and may influence glycogen synthase kinase 3 (GSK3)-dependent phosphorylation of tau (Bertram and Tanzi, 2009).
- *GALP* encodes for galanin-like peptide (GALP) and increases the risk for AD by about 10 % (Grupe et al., 2007).
- *PGBD1* encodes for piggyBac transposable element derived 1 (PGBD1) and increases the risk for AD by about 20 % (Grupe et al., 2007),
- *CLU* encodes clusterin (also known as apolipoprotein J), a glycoprotein expressed ubiquitously. Clusterin is proposed to enhance the transport of boluble Aβ from the plasma across the blood-brain barrier (Bertram and Tanzi, 2009; Eisenstein; Zlokovic et al., 1996). However, ApoE is proposed to transport Aβ in the reverse direction from the brain to the plasma (Tanzi et al., 2004). Hence, ApoE and clusterin may be important modifiers of Aβ with reference to its transport into and from the brain (Bertram and Tanzi, 2009).

Most of these genes are proposed to increase the risk for AD by 10 – 20 % (Bertram and Tanzi, 2009; Eisenstein 2011)

The importance of abnormal RNA processing in neurodegenerative disease, however, is becoming increasing clear (Anthony and Gallo, 2010). The following sections will review the importance of accurate alternative splicing and the function of RNA-binding proteins in neurodegeneration, paying particular attention to the role of aberrant splicing in tauopathies such as Alzheimer's disease (AD). Essentially, we focus on the role of specific RNA-binding proteins involved in the regulation of tau splicing which are abnormally expressed and/or hyperphosphorylated in AD.

1.2 Alzheimer's disease brain pathology

Dementia is the gradual decline in the cognitive abilities of an individual, affecting their daily life due to a series of symptoms linked to diseases/damage of the brain (Blennow et al., 2006). These symptoms include loss of cognitive functions including memory (memory loss that disrupts daily activities), thinking (challenges in planning and solving problems) and behaviour (changes in mood and personality; withdrawal fom work or social activities). Although dementia is associated with aging, it is a deviation from the normal processes in the brain of the elderly. The most common type of dementia is Alzheimer's disease (AD) affecting 1 in 20 people over the age of 65 and about 26 million worldwide (Blennow et al., 2006). The prevalence of the disease is attributed to the increase in life expectancy in industrially developed countries. The World AD Report 2010 recently estimated the cost of dementia worldwide to $640 billion (World Alzheimer's Disease International World Alzheimer Report 2010; Prof Andres Wimo and Martin Prince). The large numbers affected combined with the high cost of care and treatment for sufferers makes it an active area of research.

Alois Alzheimer was the German neuropsychiatrist who over 100 years ago (1906), described a condition of his patient, Auguste D as a disease of middle-aged women with memory disturbances as well as cognitive deficits. After the death of Auguste D, autopsy of her brain revealed neuropathological hallmarks such as extracellular, miliary bodies (now known as amyloid plaques) and intracellular, dense bundles of fibrils (now known as tangles) (Figure 1.1). At present, diagnosis of AD is based on careful neurological and psychiatric examinations as well as neuropsychological testing. However, the only clear-cut diagnosis of AD is through post-mortem examination of the brain. Over a century after its discovery, a cure for AD is still elusive with current treatment being palliative, only slowing cognitive decline temporarily (Blennow et al., 2006).

Biological changes observed in AD exist long before the onset of clinical symptoms (Bookheimer et al., 2000). Before the development of AD, some patients have a symptomatic, pre-dementia syndrome known as mild cognitive

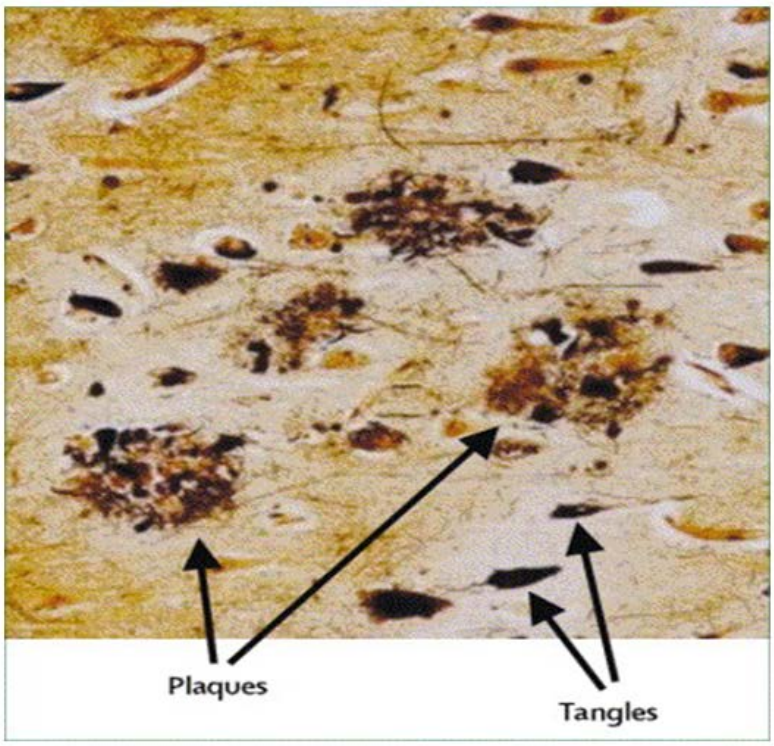


Figure 1.1. Amyloid plaques and neurofibrillary tangles in an AD brain (Blennow et al., 2006). Neurofibrillary tangles are intracellular aggregates consisting of the hyperphosphorylated form of the microtubule associated protein, tau, while amyloid plaques exist as extracellular deposits of amyloid β protein surrounding neurons.

impairment which may lead to AD development later (Flicker et al., 1991). During the progression of AD, symptoms observed include personality and behavioural changes in addition to problems recognising friends and family members.

The distinctive pathological features of AD are accumulation of protein aggregates in the form of extracellular amyloid (neuritic plaques) and intracellular neurofibrillary tangles (NFTs) (Figure 1.1) as well as a remarkable decrease in brain volume due to neuronal loss (Blennow et al., 2006). Immunochemical and electron microscopic studies of extracellular plaques identified amyloid fibres as the core of amyloid plaques (Terry et al., 1964). Thereafter, the amyloid fibres were shown to consist of β-amyloid (Aβ) peptides (Glenner and Wong, 1984; Glenner et al., 1984) derived from the cleavage of the transmembrane glycoprotein amyloid precursor protein (APP) (Kang et al., 1987)). Insoluble Aβ accumulates in the extracellular matrix into amyloid deposits, surrounded by axonal and dendritic processes. In an AD patient's brain, amyloid plaques are predominantly found throughout the cortex, including the isocortex, frontal, temporal and occipital lobes (Braak and Braak, 1991).

NFT pathology progression during AD

NFTs in AD are intracellular aggregates consisting of paired helical filaments (PHFs) (Kidd, 1963). Particularly, PHFs are made up of filaments twisted around each other and therefore referred to as 'helical'. PHFs are made up of the microtubule-associated protein, tau in its hyperphosphorylated form (Grundke-Iqbal et al., 1986).

NFT pathology progresses in six stages with tangles first appearing in the pyramidal neurons of the transentorhinal cortex which then spread to the entorhinal cortex, the hippocampus and surrounding limbic regions such as the amygdala before reaching association areas of the cerebral cortex (Braak and Braak, 1991; Selkoe, 2001). This progression of NFT in brain regions is the basis of 'Braak staging'. Brain regions including the motor cortex, basal ganglia and cerebellum are relatively unaffected in AD (Braak and Braak, 1991).

Plaques and tangles spread throughout the cortex as AD progresses (Braak and Braak, 1991, 1992). However, the speed of AD progression is diverse and depends on the age of onset and the presence of other health conditions. There are three prominent stages in AD progression:

- **Preclinical AD, Braak stages I - II** – changes may start 20 years before diagnosis. However, from this stage, further decline is unavoidable. In the earliest stages, before symptoms can be detected, plaques and tangles are formed in areas of the brain involved in speaking and understanding (Braak and Braak, 1991).

- **Mild to moderate AD, Braak stages III - IV** – this stage lasts between 2 - 10 years. In these stages, brain regions essential in memory, thinking and planning develop more plaques and tangles than observed in the early stages. Hence, memory and thinking problems arise that are serious enough to interfere with work or social life. These individuals may also get confused, experience difficulty in articulating themselves or organizing their thoughts. Most AD patients are diagnosed at this stage (Braak and Braak, 1991).

- **Severe AD, Braak stages V - VI** – this stage lasts from 1 - 5 years (www.alz.o. In advanced AD, most of the cortex including the occipital cortex is seriously damaged and the brain shrinks dramatically due to widespread neuronal death (Braak and Braak, 1991). Individuals lose their ability to communicate, to recognize family and loved ones and to care for themselves.

1.3 The microtubule-associated protein, tau

The cytoskeleton is vital for the maintenance of cellular morphology, intracellular organisation as well as interaction with surrounding cells. It consists of three groups of filaments; microfilaments, intermediate filaments and microtubules. The microtubule is a cylindrical protein polymer made up of tubulin subunits (α- and

β-tubulin) (Kirschner and Mitchison, 1986). Stabilisation of the microtubule is dependent on accessory proteins referred to as microtubule associated proteins (MAPs) which structurally organise the microtubule in the cell when required (Ramirez et al., 1999). Microtubules form the transport machinery within cells. They are involved in the maintenance of neuronal morphology, formation of axonal and dendritic processes in addition to cellular trafficking (Hanger et al., 2009; Matus et al., 1990). For example, microtubules determine the destination of cellular organelles as well as intracellular transport of proteins and vesicles from the cell body to the synapse of neurones.

The microtubule associated protein, tau was discovered in 1975 after isolation from tubulin (Weingarten et al., 1975). In the absence of tau *in vitro*, tubulin exists as a dimer of α- and β-tubulin subunits which did not assemble into microtubules (Weingarten et al., 1975). However, in the presence of tau, tubulin polymerises to form microtubules (Weingarten et al., 1975). Hence, tau acts as a connecting protein for microtubule subunits by binding through its microtubule-binding domain (Figure 1.2) thereby promoting microtubule assembly and stability. Tau is predominantly localised in the axon of neurones. Tau exists in a dynamic balance between phosphorylated and dephosphorylated forms (Johnson and Stoothoff, 2004). Depending on its phosphorylation state, tau modulates microtubule assembly and stability which are essential for axonal growth and transport of proteins such as tubulin that makes up the axonal cytoskeletal polymers.

Structure and alternative splicing of the *MAPT* gene

The *MAPT* gene encodes human tau and spans 134 kb on chromosome 17 at position 17q21 (Neve et al., 1986). There are two haplotypes of the *MAPT* gene, H1 and H2 (Stefansson et al., 2005). H1 and H2 are identified by regions with linkage disequilibrium between several polymorphisms over the entire *MAPT* gene both of which do not recombine because of their opposing orientation to each other (Pittman et al., 2004). An invariant H2 haplotype differs from the H1 haplotype by the presence of eight haplotype tagging single nucleotide polymorphisms (htSNP) which are variations at single positions in the DNA

sequence and a 238 bp deletion in intron 9 (Pittman et al., 2004). The H1 haplotype is further divided into three sub-haplotypes; H1a, H1b and H1c due to the presence of further single nucleotide polymorphisms (SNPs) that are distinguished only on a H1 background (Pittman et al., 2005).

The *MAPT* gene consists of sixteen exons with six alternative exons including exon 2, 3, 4a, 6, 10 and 13. The first and last exons, exon -1 and 14 are transcribed but not translated into protein (Figure 1.2) (Andreadis et al., 1992) (Andreadis, 2005). Exons 2, 3 and 10 of the *MAPT* gene are alternatively spliced to produce six different isoforms of the tau protein in the adult human brain (Figure 1.2). Tau transcripts containing exons 2, 3 and 10 are present in different combinations as well as being regulated in a developmental- and tissue-specific manner (Andreadis, 2005).

Alternative splicing of a gene is the joining of exons in different patterns. It involves the inclusion or exclusion of an exon to generate different forms of the mRNA transcript from the same pre-mRNA. For example, tau exon 10 may be included or excluded in the mRNA transcript (Figure 1.2) (Keren et al., 2010).

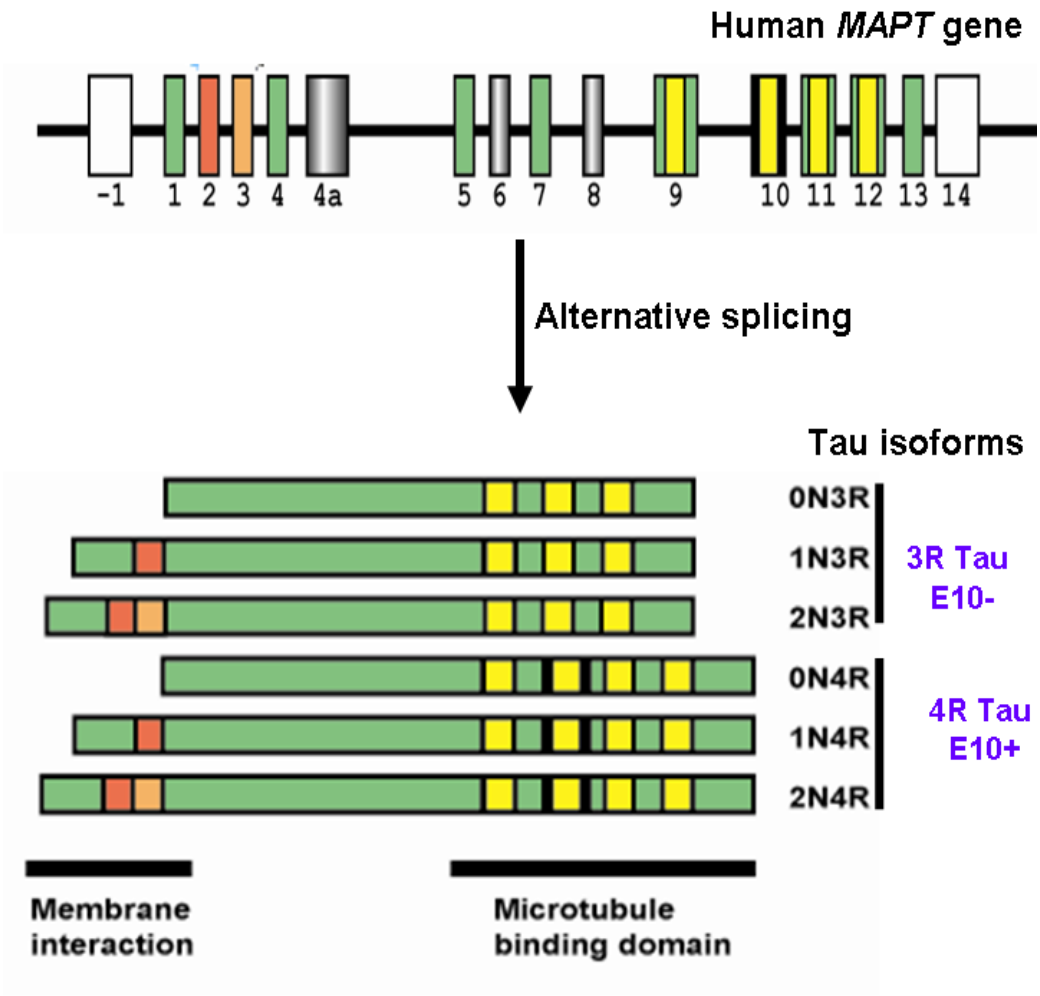


Figure 1.2. Alternative splicing of the *MAPT* gene and the six tau isoforms (from (Gallo et al., 2007)).

Alternative splicing of exons 2, 3 and 10 give rise to six isoforms from a single gene in the brain. The presence or absence of exons 2 and 3 in the N-terminus and exon 10 in the C-terminus generates different tau isoforms. Exon 10 inclusion produces tau isoforms containing four microtubule-binding repeats (4R tau) while its exclusion produces tau isoforms containing three microtubule-binding repeats (3R tau).

1.3.1 Alternatively spliced tau exons

1.3.1.1 Tau exon 2 and 3

The amino-terminal of tau proteins contains a 29- or 58-amino acid insert encoded for by exon 2 alone (1 N-terminal insert) and exons 2 and 3 (2 N-terminal inserts) respectively (Goedert et al., 1989a) (Figure 1.2). These N- terminal inserts of tau are expressed in an adult-specific manner. Exon 2 can be expressed independently in the tau mRNA transcript; however exon 3 is never expressed independently (Li et al., 2003). By default, the expression of exon 3 is exclusion and dependent on the presence of exon 2 (Andreadis, 2005; Andreadis et al., 1995) while the default splicing pattern of exon 2 is inclusion (Andreadis et al., 1995). Tau interacts with neuronal plasma membrane through its N-terminal and is proposed to mediate the microtubule-plasma membrane interaction (Brandt et al., 1995).

1.3.1.2 Tau exon 10

At the carboxyl-terminal, tau contains either 4 or 3 microtubule-binding repeats (R) encoded for by exons 9 - 12 (Andreadis, 2005; Goedert et al., 1992b). Alternative splicing of tau exon 10 encodes for the second microtubule-binding repeat (R) of tau (Figure 1.2). This divides the six tau isoforms generated after alternative splicing into two groups, depending on the inclusion or exclusion of exon 10, designated as tau with four (4R) or three (3R) microtubule-binding repeats respectively (Figure 1.2). The expression of 4R and 3R tau containing transcripts is developmentally regulated with only 3R tau being expressed during embryonic development while 4R tau is expressed in an adult-specific manner (Goedert et al., 1989a). The 4R/3R ratio is approximately one in normal human adult brain (Hutton et al., 1998), while only 4R tau is expressed in the brain of adult rodents (Kosik et al., 1989). The presence or absence of tau exon 10 in the mRNA transcript modulates tau's affinity for microtubules, an important role for tau in neurons. Also, the lack of exon 10 (3R tau) increases neuronal plasticity and reduces binding to microtubules which are essential during development (Goedert et al., 1998).

1.3.2 Tau function and distribution

Translation of the six tau mRNA isoforms generates different tau proteins with several physiological roles in the cell. These include microtubule-dependent and microtubule-independent functions:

- **Polymerisation and stabilisation of microtubules.** The C-terminal of the tau protein has three or four microtubule-binding repeats (Figure 1.2) that allow for interaction with tubulin subunits to promote assembly of microtubules, growth and nucleation (Brandt and Lee, 1993). The microtubule is in a state of dynamic instability, with alternating phases of growth and rapid shrinkage (Mitchison and Kirschner, 1984). Therefore, microtubules exist in a dynamic state of change between polymerisation and depolymerisation, depending on the cellular requirement (Kirschner and Mitchison, 1986). This regulates the trafficking of molecules including cellular organelles such as mitochondria along the microtubule cytoskeleton (Howard and Hyman, 2009).

- **Neurite outgrowth.** The dynamic behaviour of microtubules is regulated differentially in different parts of the neuron. In neurons, tau is predominantly localised in the axons with the largest amounts found at the distal end, close to the growth cone, in developing neurons (Black et al., 1996). Microtubules at the distal end of the axon as well as growth cones are the most dynamic (Ahmad et al., 1993). The involvement of tau during neurite outgrowth was demonstrated when PC12 cells treated with nerve growth factor (NGF), showed an increased expression of tau which correlated with neurite outgrowth (Drubin et al., 1985). However, treatment of PC12 cells with antisense tau oligonucleotides inhibits tau expression and revealed a reduction in neurite formation and the number of microtubules (Black et al., 1996; Caceres and Kosik, 1990; Caceres et al., 1991).

- **Signal transduction**. Tau exons 2 and 3 may interact with specific membrane compartments involved in signal transduction (Brandt et al., 1995). The src family of tyrosine kinases such as fyn have a src homology 3 (SH3) domain which interacts with the proline-rich domain (pro-lys-ser-pro sequence) of tau (Lee, 2005; Usardi et al., 2011). Tau was identified as a substrate of fyn through co-transfection experiments of tau and fyn in COS cells. Tau is phosphorylated by fyn at tyr18 in transfected cells in a manner that is independent of tau's microtubule association properties (Lee, 2005; Usardi et al., 2011). Furthermore, tau-fyn complexes are located underneath the plasma membrane and associated with the actin cytoskeleton (Lee et al., 1998). Hence, tau may be important in src family tyrosine kinase signalling processes that modify cell shape through their activity on the actin cytoskeleton. Tyrosine phosphorylation of tau suggests its involvement in neuronal signal transduction (Usardi et al., 2011). This may account for other functions of tau in cell signalling besides its main role in microtubule assembly (Lee, 2005).

- **Axonal transport**. The transport of vesicles and organelles in the antetrograde direction, from the cell body to the synapse; and retrograde direction from the synapse to the cell body requires a stable axonal transport network that involves microtubules, stabilised by tau (Mandelkow et al., 2003). Tau competes with motor proteins involved in the transport of cargo along the microtubule tracks including kinesin and kinesin-like motors (Dixit et al., 2008). Overexpression of tau interferes with the binding of these motor proteins and inhibits kinesin dependent trafficking of mitochondria, endoplasmic reticulum and vesicles (Dixit et al., 2008) (Ebneth et al., 1998). In addition, high concentrations of tau inhibit kinesin dependent transport of filaments and Golgi-derived vesicles and peroxisomes (important for detoxification of H_2O_2) to neurites which makes them susceptible to oxidative stress (Stamer et al., 2002). Furthermore, increased expression of tau inhibits the transport of APP causing its accumulation in the cell body (Stamer et al., 2002).

1.4 Tau pathology in neurodegeneration

A group of neurodegenerative dementias and movement disorders called tauopathies have tau as the major component of neurofibrillary tangles (NFTs) in which tau is abnormally phosphorylated and assembled into filamentous aggregates. Tauopathies include:

- Alzheimer's Disease (AD)
- Frontotemporal dementia with parkinsonism linked to chromosome 17, (FTDP-17)
- Pick's Diseases (PiD)
- Progressive Supranuclear Palsy (PSP)
- Coritcobasal Degeneration (CBD)
- Myotonic Dystrophy (DM)

Tau aggregates in tauopathies may be in the form of intracellular filamentous deposits in the brain including NFTs, neuropil threads and glial tangles (Spillantini and Goedert, 1998). However, the morphology, tau content and localisation of the tau deposits are dependent on the specific tauopathy. These aggregates consist of paired helical filaments (PHFs) and straight filaments (SFs) (Crowther et al., 1992). Biochemically in AD, these deposits may consist of all six tau isoforms present in adult human brain (Goedert et al., 1989a). The H1 sub-haplotypes, H1b and H1c have a high degree of association with CBD and PSP tauopathies (Myers et al., 2005; Myers et al., 2007; Pittman et al., 2005; Pittman et al., 2004) while the H1c haplotype is associated with an increased risk for AD (Myers et al., 2005).

In these diseases, tau's binding to microtubules is impaired which leads to destabilisation of neuronal microtubules and degeneration of dendrites, axons and synapses before cell death (Ballatore et al., 2007). The NFT pathology in these tauopathies correlates with neuronal loss and cognitive decline due to alterations in the tau protein (Spires-Jones et al., 2009).

Abnormal levels of tau phosphorylation are the most prominent hallmark of tau in tauopathies (Braak and Braak, 1992; Goedert and Spillantini, 1990). In addition, mutations in *MAPT* gene highlighted the direct link between tau and tauopathies (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini and Goedert, 1998). Moreover, abnormal splicing of tau exon 10 is an important pathological event in FTDP-17. The complex regulation of tau exon 10 is detailed below.

1.4.1 Tau phosphorylation

Tau is usually phosphorylated in the cell but its activity is regulated by the degree of phosphorylation (Tanaka et al., 1998). Tau phosphorylation is achieved through the activity of several kinases and phosphatases. Amongst these are two prominent kinases, glycogen-synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (cdk5) (Hanger et al., 1992) (Hanger et al., 2009). Appropriate phosphorylation of tau enhances its physiological roles in the cell including neurite outgrowth, axonal transport and microtubule dynamics (Biernat and Mandelkow, 1999); (Tatebayashi et al., 2004); (Cho and Johnson, 2004).

In pathological conditions such as AD, tau becomes hyperphosphorylated due to an imbalance in the activity of kinases that phosphorylate tau and phosphatases that de-phosphorylate tau. This results in a tendency of hyperphosphorylated tau to loose its microtubule association and form insoluble fibrils and tangles within neurons (Abraha et al., 2000; Jameson et al., 1980). Whether tau is phosphorylated in NFTs before or after aggregation is unknown. Hyperphosphorylated tau sequesters normal tau as well as other MAPs thereby causing depolymerisation of microtubules and impairing axonal transport which compromises synaptic function (Buee et al., 2000) and may even increase cell death (Fath et al., 2002). All six tau isoforms are present and hyperphosphorylated in AD brain and aggregate into PHFs in tangles (Goedert et al., 1992a; Goedert et al., 1989b). There are thirty-nine phosphorylation sites identified in PHFs by mass spectrometry in addition to six sites detected with phospho-specific antibodies (Goedert et al., 1992a; Grundke-Iqbal et al., 1986; Hanger et al., 2009).

1.4.2 Alternative splicing

In eukaryotic cells, splicing is a well-defined RNA processing event. After transcription of a protein-coding gene, the mRNA transcript undergoes several post-transcriptional modifications including capping, splicing and polyadenylation which may occur co-transcriptionally or consecutively in the nucleus (Maniatis and Tasic, 2002).

Splicing of pre-mRNA involves the accurate removal of non-coding sequences (introns) and the simultaneous ligation of coding sequences (exons) to form a mature messenger RNA (mRNA) transcript (Long and Caceres, 2009). Alternative splicing allows for the production of multiple mRNA from a single gene. As a result, the different mRNA are translated into different protein isoforms, with varying location and function (Andreadis et al., 1992; Douglas and Wood, 2011; Modrek and Lee, 2002). This also modulates the expression of different protein isoforms in a tissue- and developmental-dependent manner, depending on the requirements of the cell (Black et al., 1996; Ladd et al., 2004).

1.4.2.1 Regulation of alternative splicing

The spliceosome catalyses the splicing reaction to remove introns through accurate recognition of *cis*-acting elements as well as donor and acceptor sites (5'-, 3'-splice sites respectively) found at the intron-exon boundaries of pre-mRNA (Figure 1.3) (Mount, 1982). Also present within the intron is the branch point (A) and polypyrimidine (polyY) tract which are important as early entry point of the spliceosome and removal of the intron (Figure 1.3). In addition to these splicing signals, splicing regulation involves regulatory *cis*-acting elements in both exons and introns. These are divided into four categories: exonic splicing enhancer (ESE), exonic splicing silencer (ESS), intronic splicing enhancer (ISE) and intronic splicing silencer (ISS) (Figure 1.3).

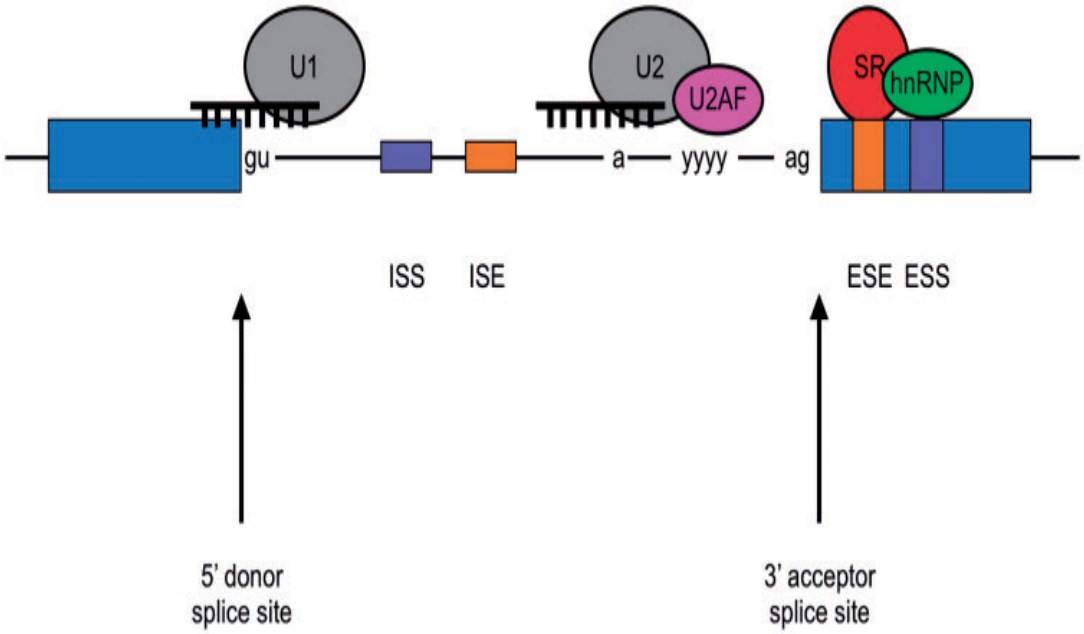


Figure 1.3. Control elements regulating splicing. (from (Douglas and Wood, 2011)).

Complementary base pairing of U1 and U2 snRNPs to the 5' splice site and branch point respectively occurs through their binding to loose consensus sequences. The polypyrimidine tract serves as a binding site for U2AF (U2 auxillary factor). SR proteins bind to ESEs and exert a positive effect to increase splice site use, while hnRNPs bind to ESSs, exerting a negative effect on splice site use.

Cis-acting elements serve as binding sites for *trans*-acting factors (RNA-binding proteins/splicing factors) that promote or inhibit the inclusion of an alternative exon (Douglas and Wood, 2011).

The alternative splicing reaction depicts that splicing factors bind to enhancer elements within the pre-mRNA to promote exon inclusion for instance, a group of proteins rich in serine and arginine residues (SR proteins) while splicing factors that repress exon inclusion bind to silencers; including members of the heterogeneous nuclear ribonucleoproteins family (hnRNPs) (Figure 1.3) (Douglas and Wood, 2011). Nevertheless, SR proteins and hnRNP proteins may repress or stimulate splicing respectively. For example, in the adenovirus infection, the SR protein, ASF/SF2, binds to an intronic repressor element near the branch point of the adenovirus IIIa pre-mRNA and inhibits the binding of a small nuclear ribonucleoprotein (snRNP), U2 snRNP (to prevents the use of the 3' splice site) (Douglas and Wood, 2011; Kanopka et al., 1996). The presence of regulatory proteins including SR proteins and hnRNPs on splice site signals through their RNA- and protein-binding domains mediates alternative splicing (Douglas and Wood, 2011).

Regulation of tau pre-mRNA alternative splicing

The alternative splicing of tau exon 2, 3 and 10 is accurately regulated due to interaction between *trans*-acting factors (splicing factors) and *cis* regulatory elements. Several studies have investigated the *trans*-acting factors involved in the regulation of tau splicing (Table 1.1). These include members of three splicing factor families: hnRNP proteins, SR proteins and and CELF {CUG-BP1 (CUG Binding Protein) and ETR-3 [(ELAV (Embryonic Lethal, Abnormal Vision) type RNA-binding protein] Like Factors} proteins.

Although the default splicing pattern for exon 2 is inclusion, most of the splicing factors tested *in vitro* inhibit its inclusion (Table 1.1). For instance, SRp30c and SRp55 significantly inhibit exon 2 splicing while CELF4 activates it (Li et al., 2003). Hence, exon 2 may be regulated primarily by inhibition (Andreadis, 2005). In contrast, the default splicing pattern of exon 3 is exclusion and never independent of exon 2 (Andreadis et al., 1995).

Table 1.1. Splicing factors influence on three tau alternative exons 2, 3 and 10 (Andreadis, 2005);(Qian et al., 2011a)

Regulator	**Exon**		
	2	**3**	**10**
SRp20	N	I	(I)
ASF	N	I	(I)
SRp30c	I	I	I
SC35	(I)	I	A
SRp40	(I)	*	A
9G8	N	nd	I
SRp55	I	A	I
SRp75	N	A	I
U2AF	N	*	I
PTB	I	*	I
hnRNPA1	N	I	N
hnRNPG	N	nd	I
Tra2-β	I	I	A
CELF3	(I)	I?	A
CELF4	A	I?	A

A=activator, I=inhibitor, N=no effect, ( ) =weak action, nd=not determined, *=construct or cell type specific.

Tau exon 10 is included in the tau mRNA transcript by default and its expression is highly regulated by its flanking exons; exons 9 and 11, which function to promote and inhibit exon 10 inclusion respectively (Gao et al., 2000). The regulation of tau exon 10 splicing involves ESE sequences at the 5' and 3' ends of exon 10 (Figure 1.4) (D'Souza and Schellenberg, 2005) as well as ESS sequences; both of which are indispensable for accurate splicing (D'Souza and Schellenberg, 2002, 2005). In addition, there are well-characterised weak 5' and 3'splice sites, a polyY tract and two separate branch point sequences in intron 9 and a bipartite regulatory element consisting of an intronic splicing silencer (ISS) and an intronic splicing modulator (ISM) in intron 10 (D'Souza and Schellenberg, 2002, 2005). ESEs present within exon 10 include:

- SC35-like sequence
- PPE (polypurine enhancer) element which enhances the 5' and 3' splice sites (D'Souza and Schellenberg, 2002).

- A/C-rich (ACE) enhancer, that works with the PPE enhancer to neutralize an inhibitory ISS element in intron 10 (D'Souza and Schellenberg, 2002).

- ESE element at the 3' end of exon 10 between an 18 nucleotide ESS element and the 5' splice site (D'Souza and Schellenberg, 2005).

The regulation of exon 10 splicing is paramount for maintaining the appropriate 4R/3R ratio in the adult human brain (Andreadis, 2005; Myers et al., 2007). There are two models for the regulation of tau exon 10 splicing:

- In the linear model, different concentrations of splicing factors compete between the two ISS elements within intron 10 and the 5' splice site (Figure 1.4) (D'Souza and Schellenberg, 2002). Although SR proteins are splicing enhancers, they can also act as repressors depending on where they bind in the pre-mRNA (Kanopka et al., 1996). For example, the SR protein, 9G8 binds to the ISS in intron 10 and inhibits exon 10 inclusion (Gao et al., 2007).
- A putative stem loop structure is proposed to regulate exon 10 splicing (D'Souza et al., 1999; Grover et al., 1999).

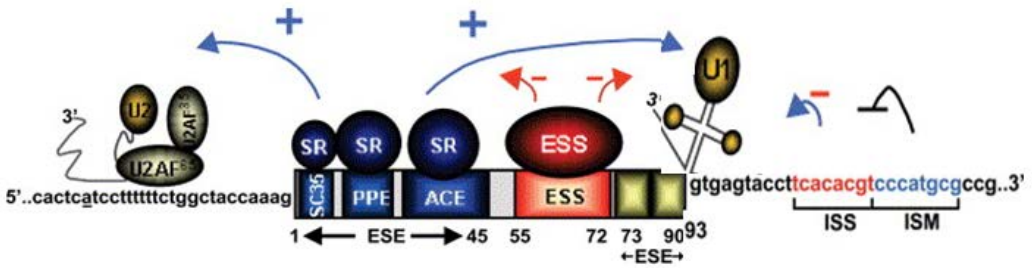


Figure 1.4. Tau exon 10 splicing regulation (adapted from (D'Souza and Schellenberg, 2005))

Tau exon 10 alternative splicing is regulated by both positive (blue arrow) and negative (red arrow) interactions between *trans*-acting splicing factors and *cis*-acting elements within the pre-mRNA. SR proteins bind to ESE elements in exon 10 to stabilise the interaction between U1 and U2 snRNP with the 3' and 5' splice sites respectively. Several ESE elements counteract weak 3' and 5' splice sites to promote exon 10 inclusion.

Key:

A/C-rich enhancer (ACE);

Polypurine enhancer (PPE);

Exonic splicing silencer (ESS); Exonic splicing enhancer (ESE);

Intronic splicing silencer (ISS);

Intronic splicing modulator (ISM)

1.5 The importance of alternative splicing

Alternative splicing occurs in over 90 % of human genes (Pan et al., 2008; Wahl et al., 2009). In addition to this, at least 15 % and probably 50 % of human genetic diseases are due to mutations in either the consensus splice sites or the variable *cis*-acting elements which results in aberrant splicing (Cartegni et al., 2002; Lopez-Bigas et al., 2005). Genome-wide analysis elucidated that most of the tissue-specific alternatively spliced isoforms are expressed in the brain (Shai et al., 2006).

Splice sites are loosely conserved in the mammalian genome and mutations at these sites results in abnormal splicing either through exon skipping or the recognition of cryptic splice sites that are not usually used during splicing (Cartegni et al., 2002). Moreover, disruption of the alternative splicing process can cause deficiency in cellular and tissue requirements, leading to disease.

1.5.1 Aberrant splicing of tau results in FTDP-17

In 1998, genetic studies on the *MAPT* gene provided the most convincing evidence for the involvement of tau in disease. Dominant mutations (alterations in the DNA sequence) in the *MAPT* gene were linked to the rare dementia, FTDP-17 (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). FTDP-17 is an autosomal-dominant disease with variable clinical and neuropathological characteristics. Symptoms observed in patients can be behavioural (personality changes and reduced speech output), cognitive (loss of executive functions) and motor (bradykinesia) changes (Poorkaj et al., 1998).

Neuropathologic changes in the brain are abundant in cortical and subcortical areas of FTDP-17 cases (Foster et al., 1997); including frontotemporal atrophy, occasionally with atrophy of the amygdala, basal ganglion, and substantia nigra (Poorkaj et al., 1998).

To date, there are over 30 mutations in the *MAPT* gene associated with FTDP-17. Dominant mutations in the *MAPT* gene cause tau dysfunction through one or more distinct mechanisms (Foster et al., 1997; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). *MAPT* mutations that cause FTDP-17 are divided into two groups.

- The first group includes missense (single amino acid substitutions), deletion, or silent mutations. Some missense mutations decrease the microtubule binding ability of tau to promote microtubule assembly (Hasegawa et al., 1998; Hong et al., 1998).

- The second group includes splice site and intronic mutations located in or around the microtubule-binding domains of tau (Figure 1.5) (Hutton et al., 1998). These mutations alter the regulation of tau exon 10 pre-mRNA splicing causing a change in the relative proportion of 4R and 3R tau isoforms and promote tau filament formation (Foster et al., 1997; Hong et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998).

1.5.1.1 Missense mutations in FTDP-17

About 50 % of mutations in FTDP-17 are missense mutations. These mutations directly alter tau function and disrupt microtubule binding capacity (D'Souza and Schellenberg, 2005). The rationale behind this is that most missense mutations are in the C-terminal end of tau, within or around the microtubule-binding domain (Gallo et al., 2007). For instance, the missense mutation, P301L (substitution of proline to leucine within tau exon 10) causes the PGGG motif in this microtubule binding repeat to become LGGG (Hutton et al., 1998). The P301L mutation affects only tau transcripts containing exon 10, causing a 90 % reduction in the rate of tubulin polymerisation (Hasegawa et al., 1998). Furthermore, tau aggregates from patients with the P301L mutation mainly contain 4R tau isoforms (Hutton et al., 1998).

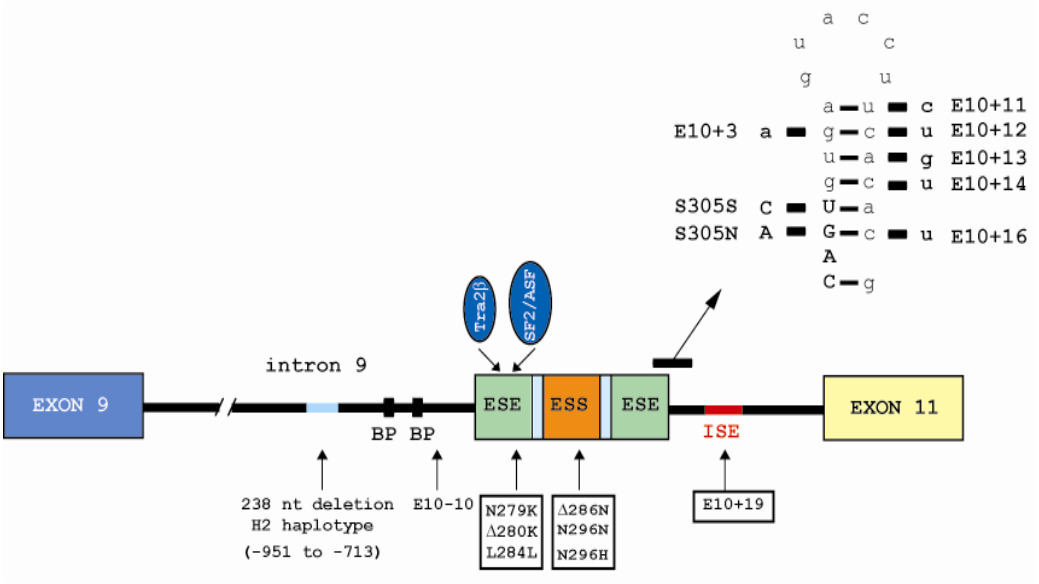


Figure 1.5. Dominant *MAPT* mutations in FTDP-17 and elements regulating alternative splicing of exon 10 (from (Gallo et al., 2007)).

Trans-acting factors and *cis*-acting elements regulating tau exon 10 splicing are shown with the predicted stem loop structure at the exon 10/intron 10 boundary (uppercase is exonic sequences, lowercase is intronic sequences). *MAPT* mutations implicated in FTDP-17 which change tau 4R/3R isoform are indicated. Most of these mutations are present within the predicted stem loop structure or within exon 10.

1.5.1.2 Intronic and splice site mutations in FTDP-17

Mutations within the exon 10/intron 10 junction of the *MAPT* gene destabilize the putative stem loop structure credited with the regulation of tau exon 10 alternative splicing (Figure 1.5) (D'Souza et al., 1999; Grover et al., 1999; Hutton et al., 1998). Although silent and intronic mutations of FTDP-17 do not affect the amino-acid sequence of the tau protein, they eventually affect exon 10 splicing and tau function (D'Souza and Schellenberg, 2005).

Silent or intronic mutations alter tau splicing by disrupting either the use of the 5' splice site or exonic sequences (PPE, ACE or ESS) in exon 10. This results in alteration of the 4R/3R tau ratio in FTDP-17 to favour 4R tau (at a 2-3 to 1 ratio of 4R/3R) as opposed to 1 in normal adult human brain (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). The fact that this alteration of the normal 4R/3R tau ratio was sufficient to cause FTDP-17 shows the importance of correct alternative splicing regulation of tau in the human brain (Andreadis, 2005; Hutton et al., 1998; Spillantini et al., 1998). In addition, RT-PCR analysis of four FTDP-17 brains from patients with 5' splice site mutations revealed a two-six fold increase in 4R tau transcripts compared to controls (Hutton et al., 1998). Similarly, the 4R/3R tau ratio in the frontal and temporal lobe was increased by up to six-fold in FTDP-17 patients with intronic mutation at position +16 (Connell et al., 2005). Hence, intronic and/ splice site mutations result in aberrant alternative splicing pattern of tau exon 10 causing disease.

1.5.1.3 Exonic mutations in FTDP-17

There are several exonic mutations within the ESE sequences of tau exon 10 which enhance splicing of exon 10 rather than reduce microtubule assembly (Hasegawa et al., 1999). Exonic mutations that affect tau exon 10 splicing include, N279K and Del280K associated with FTDP-17 which disrupts the PPE splicing enhancer in exon 10 by adding or removing an AAG motif in the normal sequence of PPE respectively (Figure 1.5) (D'Souza and Schellenberg, 2005; Jiang et al., 2003). The N279K mutation strengthens the PPE while the Del280K mutation

abolishes it (Kondo et al., 2004). The SR-related protein tra2β (transformer 2 β) which regulates tau exon 10 splicing has a higher affinity for PPE with N279K mutation and promotes exon 10 inclusion while Del280K reduces exon 10 inclusion (D'Souza and Schellenberg, 2005; Kondo et al., 2004).

Maintaining the 4R/3R tau ratio is paramount for normal tau function because tauopathies with aberrant tau exon 10 splicing such as CBD and PSP have 4R tau as the main component of the tangles (Chambers et al., 1999; Hutton et al., 1998; Myers et al., 2007; Takanashi et al., 2002). Conversely, investigations into the relative abundance of alternatively spliced tau isoforms in sporadic AD have revealed conflicting results (Boutajangout et al., 2004; Chambers et al., 1999; Connell et al., 2005; Glatz et al., 2006; Ingelsson et al., 2006; Yasojima et al., 1999). However, two pre-mRNA encoding proteins that regulate tau exon 10 splicing, tra2β and clk2 kinase, which phosphorylates tra2β, showed altered splicing pattern in the temporal cortex of some sporadic AD patients which also had increased tau exon 10 inclusion (Glatz et al., 2006). Furthermore, the H1c haplotype of the *MAPT* gene associated with increased susceptibility for AD correlates with an increase in levels of tau transcripts containing exon 10 (Myers et al., 2005; Myers et al., 2007).

Additionally, the *MAPT* H1 haplotype is associated with tauopathies that show altered tau exon 10 splicing with increased 4R/3R tau ratio (PSP and CBD) (Baker et al., 1999; Houlden et al., 2001). On the other hand, in Pick's disease, aggregated intracellular insoluble tau consists mainly of 3R tau (Mailliot et al., 1998). These suggest that aberrant alternative splicing may contribute to the pathogenesis of tauopathies.

1.6 RNA binding proteins regulating alternative splicing

A key question in splicing and an obvious point in regulation is how external factors or proteins regulate alternative splicing. Several studies have reported the synergistic interaction between *trans*-acting factors which bind to *cis*-acting elements within the pre-mRNA transcript to regulate alternative splicing (Andreadis, 2005; Caceres et al., 1994; Kralovicova and Vorechovsky, 2007). Splicing factors are RNA-binding proteins that may be expressed ubiquitously or in a tissue-specific or developmentally controlled manner to regulate alternative splicing of specific pre-mRNAs transcripts.

Several families of RNA binding proteins are grouped on the basis of structural homology between family members, function and conservation between members or species. The following sections describe three important families of RNA binding proteins involved in the regulation of tau splicing: hnRNP proteins, SR proteins and CELF {CUG-BP1 (CUG Binding Protein) and ETR-3 [(ELAV (Embryonic Lethal, Abnormal Vision) type RNA-binding protein] Like Factors} proteins.

1.6.1 The heterogeneous nuclear ribonucleoproteins (hnRNP) family

Mammalian splicing regulators include the family of hnRNPs which consist of a diverse group of proteins that share a modular structure and RNA-binding motifs and are expressed exclusively in the nucleus (Gorlach et al., 1993; Hanamura et al., 1998). Members of the hnRNP family have three RNA-binding domains:

- RNA recognition motif (RRM)

- RGG Domain – containing tripeptide arginine-glycine-glycine repeats (Gorlach et al., 1993).

- KH Domain – ‘K homology’ domain discovered in the hnRNP K protein contains a central IGXXG motif that spans about 40 residues (Gorlach et al., 1993).

In the past, hnRNP proteins were seen as inhibitors of splicing activity; prominent examples include hnRNP A1, hnRNP I and PTB (polypyrimidine tract-binding protein) which interfere with the binding of U1 snRNP and U2AF to pre-mRNA (Gorlach et al., 1993; Martinez-Contreras et al., 2007). hnRNP proteins characteristically inhibit alternative splicing by antagonising the binding of SR proteins to pre-mRNA (Hanamura et al., 1998). hnRNP A1 interferes with the interaction of SR proteins to regulate the alternative splicing of c-src exon N1 (Rooke et al., 2003). However, it recently emerged that hnRNPs both inhibit and promote alternative splicing (Martinez-Contreras et al., 2007). Additionally, hnRNP proteins have been implicated in several roles during RNA metabolism such as pre-mRNA transport, transcription, RNA stabilisation and RNA editing (Gorlach et al., 1993; Krecic and Swanson, 1999).

1.6.2 SR proteins

Serine and arginine-rich (SR) proteins are a group of closely related RNA-binding proteins classified based on their structural features (Roth et al., 1991). SR proteins are part of the spliceosome and are actively involved in the regulation of mammalian alternative splicing (Twyffels et al., 2011; Zahler et al., 1992). There are nine human SR proteins encoded by nine genes with regions of similarity (Table 1.2) (Figure 1.6) (Kramer, 1996; Long and Caceres, 2009) based on the following criteria;

- The presence of at least one RNA recognition motifs (RRM) at the N-terminal which binds to RNA (Query et al., 1989). In some SR proteins, a second RRM-like domain with RRM homology (RRMH) is present. The only exception is the SR protein, 9G8 which has a zinc knuckle motif, thought to bind to RNA (Cavaloc et al., 1994) (Figure 1.6).

- A signature RS domain enriched in serine/arginine repeats at the C-terminal (Figure 1.6).

The RRMs have a structure which enhances their interaction with multiple RNA contacts as well as being vital in determining RNA-binding specificity (Maris et al., 2005). Conversely, the RS domain, rich in alternating arginine and serine residues contributes to the varying lengths of the different members of the SR family (Caceres et al., 1994; Kramer, 1996; Manley and Tacke, 1996) (Figure 1.6). The RS domain was detected in other RNA-binding proteins referred to as SR-like proteins such as Tra2β (D'Souza and Schellenberg, 2005; Kramer, 1996). This domain is important for mediating protein-protein interactions, splicing and the nucleocytoplasmic shuttling of individual SR proteins, acting as a nuclear localisation signal (NLS) (Kramer, 1996) (Caceres et al., 1998; Sapra et al., 2009).

Table 1.2. The nine human SR proteins family members and their encoding genes (from (Shepard and Hertel, 2009)).

Name	SR protein	Chromosomal location
SFRS1	ASF/SF2/SRp30a	17q21.3-q22
SFRS2	SC35/SRp30b	17q25.1
SFRS3	SRp20	6p21.31
SFRS4	SRp75	1p35.3
SFRS5	SRp40	14q24.2
SFRS6	SRp55	20q13.11
SFRS7	9G8	2p22.1
SFRS9	SRp30c	12q24.23
SFRS11	SRp54	1p31.1

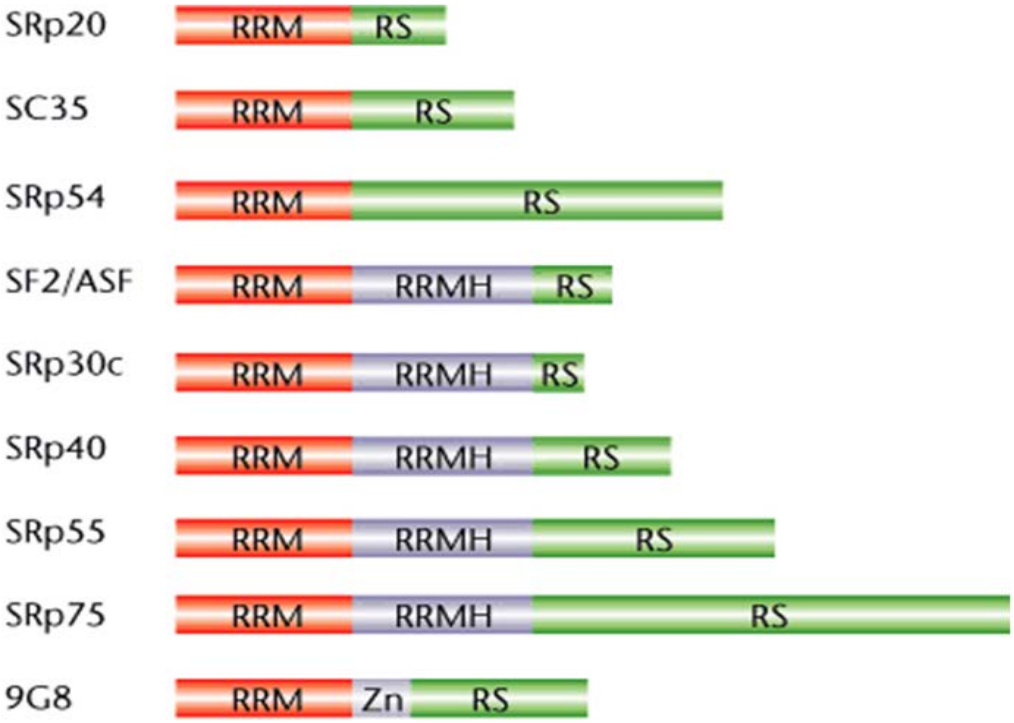


Figure 1.6. Modular structure of the nine human SR proteins (from (Shepard and Hertel, 2009)).

The nine human SR proteins are encoded by nine genes (Table 1.2). All SR proteins contain at least one RRM (orange) and an RS domain (lim.e green). Only one SR protein, 9G8 has a zinc knuckle domain (Zn) (purple). Five SR protein family members have a RRM homology (RRMH) domain (purple).

1.6.2.1 Functions of SR proteins

SR proteins have multiple roles in RNA processing including constitutive and alternative splicing (Andreadis, 2005; Zahler et al., 1992). They mediate their function either by binding directly to pre-mRNA or indirectly to components in the spliceosome during splicing to enhancer or repress nearby splice sites (Roth et al., 1991). SR proteins were first identified by their intrinsic ability to activate splicing through complementation assays with cytoplasmic S100 splicing-deficient extracts from HeLa cells containing all splicing machinery except SR proteins (Zahler et al., 1992).

1.6.2.2 Splicing and SR proteins

SR proteins are amongst the first components that interact with the pre-mRNA transcript during splicing. They have an essential role during alternative splicing, acting as regulators of mRNA metabolism (Kramer, 1996; Long and Caceres, 2009). SR proteins promote exon inclusion by binding to ESE motifs on pre-mRNA through their RRM. They bind almost exclusively to exonic regions, probably because of their role in the recruitment of other splicing factors that may stabilize their interaction with the pre-mRNA (Lin et al., 2005). The binding of SR proteins to ESE motifs enhances spliceosome assembly and promotes exon inclusion in two ways:

1. SR proteins recruit spliceosome components such as small nuclear ribonucleoproteins (snRNPs); for example U1 snRNP at the 5' splice site (Zhu et al., 2001) and U2AF at the 3' splice site (Wang and Manley, 1995) through the RS domain which mediates protein-protein interactions.

2. Competitive binding with other RNA-binding proteins for example hnRNP proteins to ESS motifs (Zhu et al., 2001). Also, two SR proteins that recognise the same ESE may compete with each other (Sanford et al., 2005a).

1.6.2.3 Role of SR proteins during splicing

SR proteins regulate alternative splicing of many pre-mRNA transcripts, by promoting the use of proximal 5' splice sites which is counteracted by hnRNP A1 and related proteins, that usually favour the use of distal 5' splice sites (Hanamura et al., 1998). Hence, SR proteins and hnRNPs play an important role in the selection of 5' splice sites *in vitro* and therefore the regulation of several pre-mRNA transcripts *in vivo*. The relative abundance of SR proteins may vary depending on the cell type, splicing condition or stage of development (Caceres et al., 1998).

Changes in SR proteins activity can lead to specific disease phenotypes or developmental defects (Long and Caceres, 2009). However, functional redundancy between members of the SR protein family is likely and can compensate for any deficiency. For example, when a single SR protein, SRp30a was knocked down in A549 cells, there was no overall consequence on cell-wide splicing events (Massiello and Chalfant, 2006). Similarly, in *Caenorhabiditis elegans*, gene inactivation using RNA interference of single SR proteins (except for ASF which is essential for nematode development) did not alter neuronal viability (Twyffels et al., 2011).

SR proteins ASF, SRp30c and Tra2β bind to the same ESE on tau exon 10, a PPE (Kondo et al., 2004; Lynch and Maniatis, 1995). Some exons including tau exon 10 contain more than one ESE recognised by different SR proteins with one having a stronger effect than the others (Lynch and Maniatis, 1995). In this case, knocking down one of the SR proteins would probably have little or no effect on splicing of the particular exon. RNAi knock down of six different SR proteins in *C. elegans* showed no phenotype. Conversely, developmental defects or death were observed when multiple SR proteins were targeted simultaneously (Longman et al., 2000). Deletion of a *Drosophila melanogaster* orthologue of human SRp55 was found to be embryonic lethal (Ring and Lis, 1994). These latter studies do indeed suggest a fundamental function for SR proteins *in vivo*.

Other SR proteins that regulate tau exon 10 splicing *in vitro* include SC35, SRp40 and SRp55 (Andreadis, 2005). In tau alternative splicing, SC35 binds to a SC35-like enhancer element in exon 10 to promote tau exon 10 inclusion (Qian et al.) while SRp40 binds to a PPE element in tau exon 10 to promote tau exon 10 inclusion (Jiang et al., 2003; Kondo et al., 2004). In contrast, SRp55 binds to an ESS to inhibit tau exon 10 inclusion *in vitro* (Andreadis, 2005; Gao et al., 2000; Wang et al., 2005).

1.6.2.4 SR protein expression is developmentally regulated

SR proteins are known to be the limiting factor in alternative splicing and show changes in their expression levels during development (Stamm et al., 1994; Zahler et al., 1992; Zahler et al., 1993). SR proteins are localised and enriched in nuclear speckles (Fu, 1995). Nuclear speckles are storage compartments for splicing factors, which respond to the level of transcription by RNA polymerase II (Misteli et al., 1997). The RS domains of SR proteins act as a localisation signal into nuclear speckles before they are recruited to splicing sites during constitutive or alternative splicing (Hedley et al., 1995).

A gradual decline of the SR protein, ASF was observed from embryonic stages to adult rat brain (Daoud et al., 1999). In addition, the level of tra2-β1 decreased from embryonic rat brain (E14, E16, E18, and E20) to postnatal stages (P5, P15 and P20) and adult rat brain (Daoud et al., 1999).

1.6.2.5 Phosphorylation regulates splicing activity of SR proteins

Serine residues within the RS domain are phosphorylation sites for kinases which modify the splicing activity of SR proteins in two ways:

1. Control of SR proteins subcellular localisation.

2. Control of protein-protein interactions of SR proteins with other RNA-binding proteins such as SR-related proteins.

All SR proteins are phosphorylated *in vivo* at their RS domain, recognised in cells, and tissues of different invertebrates and vertebrate species by a monoclonal antibody 104, mAb104 (Gui et al., 1994b; Hanamura et al., 1998; Kramer, 1996; Roth et al., 1991; Zahler et al., 1992). Several families of kinases phosphorylate SR proteins including the SR protein kinases 1 (SRPK1) (Gui et al., 1994a; Gui et al., 1994b), Clk/Sty family kinases (Colwill et al., 1996) and cdc2-like kinases (CLK) (Hartmann et al., 2001). The splicing activity of SR proteins is regulated throughout development by their phosphorylation state (Sanford et al., 2005b). However, hypo- and hyper-phosphorylated SR proteins are inactive during splicing while moderately phosphorylated SR proteins partake in splicing (Gui et al., 1994a) (Sanford and Bruzik, 2001).

SR proteins ASF, SRp20 and 9G8 constantly shuttle between the nucleus and the cytoplasm depending on their phosphorylation state. SR protein shuttling, implicates them in the export of mRNA to the cytoplasm from the nucleus (Graveley, 2000; Sanford et al., 2005a). The evidence for this comes first from the fact that both RS domain and RMMs are phosphorylated in shuttling SR proteins (Caceres et al., 1998) which may increase the association of mRNA and SR proteins. Secondly, SR proteins remain in close proximity to mRNA transcripts after intron removal. Furthermore, a protein involved in nuclear export, Tip-associated protein (TAP), interacts with 9G8 and ASF during the “hand-over” of mRNA (Hautbergue et al., 2008). SR protein kinases present in the cytoplasm phosphorylate SR proteins and activates their re-shuttling back to the nucleus (Ding et al., 2006).

1.6.3 CELF family of RNA-binding proteins

The CELF {CUG-BP1 (CUG Binding Protein) and ETR-3 [(ELAV (Embryonic Lethal, Abnormal Vision) type RNA-binding protein] Like Factors} proteins are a family of RNA binding proteins that are expressed in a developmental and tissue specific manner (Ladd et al., 2001). The tissue distribution of CELF proteins is summarised in Table 1.3.

Table 1.3. CELF protein family tissue distribution and roles in neurological diseases.

Protein	Tissue Distribution (Ladd et al., 2004)	In Disease
CELF1	Ubiquitously Expressed	Hyperphosphorylated and stabilised in DM1 patients resulting in increased activity (Kuyumcu-Martinez et al., 2007). Down-regulated in DM1 brain at RNA (Leroy et al., 2006) and protein level (Dhaenens et al., 2011).
CELF2	Ubiquitously Expressed Enriched in Skeletal Muscle and Brain	Up-regulated in muscle of SMA patients and interacts with SMN (Anderson et al., 2004). CELF2 is down-regulated three-fold in SCA3 transgenic mice compared to wild type mice (Menzies et al., 2010). Down-regulated in DM1 brain at RNA (Leroy et al., 2006) and protein level (Dhaenens et al., 2011). SNPs in *CELF2* gene are associated with late-onset AD in highest risk APOEε4 homozygotes (Wijsman et al., 2011).
CELF3	Brain Only Testes (mice) (Dev et al., 2007)	
CELF4	Ubiquitously Expressed Enriched in Skeletal Muscle	CELF4 k/o mice have a seizure phenotype (Yang et al., 2007). Unc-75 (CELF3, 4 and 5 orthologue) knock-out in *C. elegans* results in an uncoordinated movement phenotype rescued by human CELF4 (Loria et al., 2003). Down-regulated in DM1 brain at RNA level (Leroy et al., 2006).
CELF5	Brain Only	
CELF6	Brain Kidney	

CELF protein family members were identified after screening human expression sequence tag (EST) database for paralogs of CUG-binding protein, CUG-BP (also known as CELF1). There are six members of the CELF family in human ranging from CELF1 to CELF6; within the family, CELF proteins are divided into two groups, depending on sequence similarity and functional difference. The first group includes CELF1 and CELF2 (78 % identical) and the second group consists of CELF3 to CELF6 all of which have a similarity of approximately 67 % (and 43.8 % similarity to CUG-BP) (Figure 1.7) (Singh et al., 2004).

CELF1 was the first member of the CELF protein family to be discovered during investigations into molecular mechanisms of a muscle wasting disease, myotonic dystrophy (DM1). CUG-BP/CELF1 as the name suggests was found by its sequence specific binding of RNA containing CUG repeats in intronic elements or 3'-UTR (untranslated region) of pre-mRNA (Ladd et al., 2001). The second member, ETR-3, was identified from genes expressed in human foetal heart (Ladd et al., 2001). An independent search for human homologues to the *Xenopus laevis* protein BrunoL-1 identified the same six proteins, leading to the alternative classification - the 'Bruno-like' (BRUNOL) family of proteins (Good et al., 2000). Some CELF proteins are also known by specific names other than their CELF or BRUNOL nomenclature, which are summarised in Table 1.4. CELF1 and CELF2 are used interchangeably as CUG-BP1 and ETR-3 respectively (Ladd et al., 2001); (Ladd et al., 2004). The CELF nomenclature is used throughout in this thesis.

All CELF proteins have similar structures to CELF1 (Figure 1.8) (Ladd et al., 2001; Ladd et al., 2004). CELF proteins have a unique structure with two N-terminal RNA recognition motifs (RRM) domains and one C-terminal RRM separated from the N-terminal RRM by a non-conserved divergent domain whose function is unknown (Ladd et al., 2001). The structure and size of the six known members of the CELF family of proteins is shown in Figure 1.8. All CELF proteins have multiple phosphorylation sites for protein kinase C (PKC) and casein kinase II (Ladd et al., 2001). The primary sequence analysis of the divergent domain in CELF1**,** revealed a serine-rich region, phosphorylated by PKC (Figure 1.8) (Kuyumcu-Martinez et al., 2007).

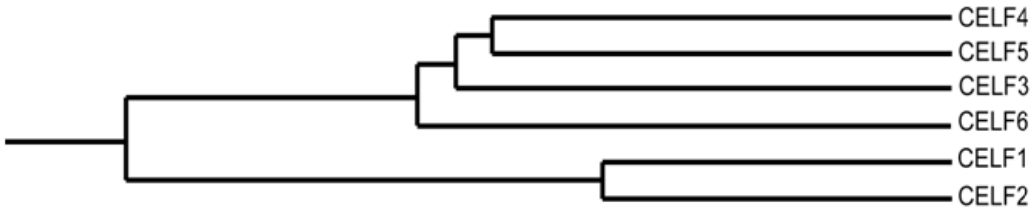


Figure 1.7. CELF proteins family and their phylogenic relationship (from (Ladd et al., 2004)).

The CELF family can be split into two sub-families based on their sequence similarity and splicing activity. The first subfamily contains CELF1 and CELF2, and the second contains CELF3, CELF4, CELF5 and CELF6.

Table 1.4. **Members of the CELF family of RNA binding proteins and their different nomenclatures**

CELF	**BRUNOL**	**OTHER**
CELF1	BRUNOL2	CUG-BP1
CELF2	BRUNOL3	ETR-3, CUG-BP2, NAPOR
CELF3	BRUNOL1	TNRC4, CAGH4
CELF4	BRUNOL4	
CELF5	BRUNOL5	
CELF6	BRUNOL6	

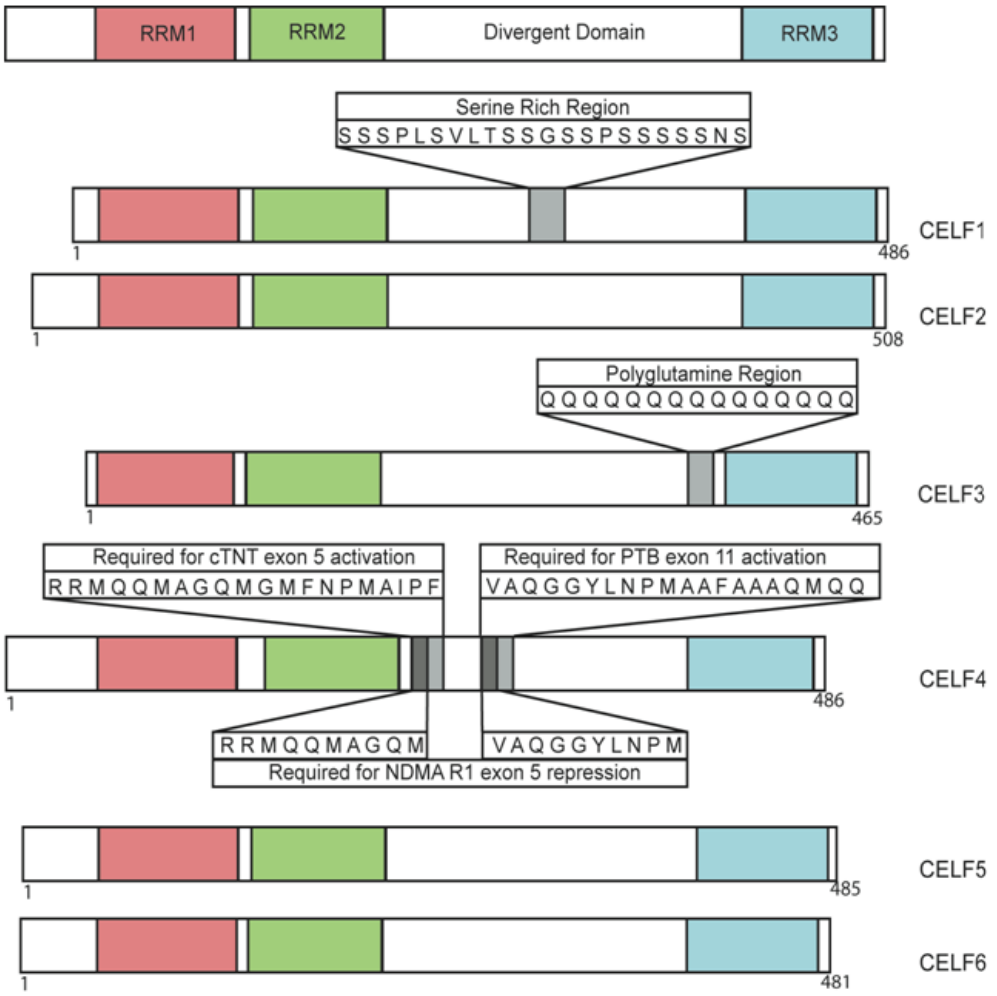


Figure 1.8. Modular structure of the human CELF protein family members (from (Gallo and Spickett, 2010)).

All members of CELF family of proteins have a similar structure that consists of three conserved RRMs, two at the N terminus, RRM1 and RRM2 (red and green), separated from the third, RRM3 (blue) at the C terminus by a divergent domain. The divergent domains of CELF1, CELF3 and CELF4 have distinctive differences within them. CELF1 has a serine rich region; CELF3 has a polyglutamine region and CELF4 has specific regions in the divergent domain that modulate the splicing activity on different pre-mRNA targets.

1.6.3.1 Functions of CELF proteins

Members of CELF protein family are involved in several post-transcriptional events including: regulation of alternative splicing, translation and mRNA localisation (Vermersch et al., 1996) (Sergeant et al., 2001) (Gallo and Spickett, 2010; Ladd et al., 2005).

The divergent domain of CELF4 is implicated in splicing activity of some specific pre-mRNA targets. These include promotion of cardiac troponin T (cTNT) exon 5 inclusion, PTB exon 11 inclusion and NDMA R1 (N-Methyl-D-aspartate receptor 1) exon 5 exclusion (Singh et al., 2004). Similarly, the divergent domain of CELF2 plays a role in alternative splicing of CFTR (cystic-fibrosis trans-membrane conductance regulator) exon 9 (Dujardin et al., 2010). CELF3 is a unique CELF protein with a 15-18 polyglutamine (polyQ) repeat in its divergent domain encoded by variable CAG repeats (Figure 1.10) (Chapple et al., 2007; Gallo and Spickett, 2010; Margolis et al., 1997). CELF3 was originally identified by a screen of candidate disease genes in cDNA libraries containing CAG repeats encoding long polyQ tracts (Margolis et al., 1997). As a result of this polyQ tract, CELF3 is also known as TNRC4 (tri-nucleotide repeat containing 4).

Some neurological disorders caused by CAG repeat expansion, include Huntington's disease (HD) and spinocerebellar ataxia type 1 (SCA1) (Rubinsztein and Carmichael, 2003) (Suzuki et al., 2008). It is unlikely that the polyQ tract in CELF3 tract will be the subject to pathogenic expansion, because it does not have a high degree of variation within the human population (Butland et al., 2007). As a result, CELF3 is not associated with any polyglutamine expansion disorders. This is because proteins with repeat CAG lengths of about 40 or more are sequestered into aggregates (Ross and Tabrizi, 2011). For instance in HD, CAG triplet repeat expansion in Huntingtin (*HTT*) gene leads to pathogenic expanded polyQ stretch in HTT protein (Lee et al., 2004; Ross and Tabrizi, 2011). Moreover, co-expressed CELF3 was not recruited into cytoplasmic huntingtin aggregates or nuclear aggregates of ataxin-1 in CHO cells (Chapple et al., 2007).

1.6.3.2 Expression of CELF proteins

The expression of CELF proteins is developmentally regulated in the heart and striated muscle (Ladd et al., 2001). All CELF proteins are expressed in adult and foetal brain (Ladd et al., 2004). In all tissues examined, there is expression of at least one or more CELF protein with the exception of CELF3 and CELF5 whose expression are restricted to the brain while CELF2 and CELF4 are developmentally regulated in striated muscle and heart (Ladd et al., 2001); (Ladd et al., 2004); (Ladd et al., 2005) (Table 1.3).

CELF proteins are localised in the nucleus and cytoplasm, conforming to their role as multifunctional proteins. CELF1 has multiple roles including regulation of alternative splicing in the nucleus; as well as mRNA translation (through its activity at the 5' end) and mRNA degradation (at the 3' end in the cytoplasm) (Timchenko et al., 1996) (Barreau et al., 2006; Paillard et al., 2003). CELF proteins are localised to the nucleus by their NLS sequences at the C-terminal, within RRM3 (Chapple et al., 2007; Ladd et al., 2001). This NLS motif located within RRM3 is conserved in all members of the CELF family; with the basic amino acid sequence KRXK.

CELF proteins bind specifically to CUG repeats in intronic elements or in the 3'-UTR (untranslated region) of pre-mRNA and mRNA transcripts (Faustino and Cooper, 2005; Ladd et al., 2001). Several CELF proteins have been implicated in the regulation of tau alternative splicing including CELF2, CELF3 and CELF4 (Chapple et al., 2007; Leroy et al., 2006; Wang et al., 2004). During alternative splicing of the *MAPT* gene, CELF proteins bind to intronic elements around alternative exons including exon 2 and 10 to govern their inclusion or exclusion in the mature tau mRNA (Andreadis, 2005). It is therefore paramount that the expression levels of these splicing factors in response to cellular stimuli is accurate to ensure precise alternative splicing of tau transcripts as well as their other pre-mRNA targets during splicing.

1.6.3.3 Functional redundancy among CELF protein family members

A prominent splicing target for CELF proteins is Cardiac troponin T (cTNT). The alternative splicing pattern of cTNT is developmentally regulated and conserved from birds to mammals (Ladd et al., 2001). The alternative cTNT exon 5 which is normally included in the embryo of cardiac and skeletal muscle is skipped in adult cardiac muscle mRNA (Ladd et al., 2001) (Barreau et al., 2006).

Alternative splicing of cTNT exon 5 generates isoforms with different contractive properties important during muscle development (Cooper and Ordahl, 1985). All CELF proteins bind to conserved intronic elements called muscle-specific splicing enhancer elements (MSEs) which contains a CUG repeat to promote exon 5 inclusion (Ladd et al., 2001). Although this splicing event is dependent on different intronic splicing enhancer (ISE) elements, CELF3 and CELF6 promote exon 5 inclusion in the presence of a muscle-specific ISE element (MSE2) found at the proximal region flanking exon 5 while other CELF proteins require several MSE elements (Ladd et al., 2001).

Furthermore, all regions within CELF proteins are implicated in the splicing regulation of cTNT exon 5. These include sequences within RRM1 and RRM2 of CELF2 as well as regions within the divergent domain and RRM1 of CELF4 (Singh et al., 2004). Altogether, these findings suggest that different ISE elements in addition to domains within CELF proteins are indispensable for splicing activity of the same exon which indicates functional redundancy among CELF family members.

An orthologue of CELF3, 4 and 5 in *Caenorhabditis elegans*, *unc*-75, is neuron specific and involved in neurotransmission where it regulates neuron-specific alternative splicing (Loria et al., 2003). *unc*-75 null mutants have a motor phenotype that is rescued by human CELF4 (Loria et al., 2003). These studies illustrate the importance of CELF proteins in maintaining neuronal function.

Similarly, the generation of mouse models deficient in specific CELF proteins, has helped to elucidate the role of members of the CELF family during alternative splicing. In mice lacking CELF1, growth is retarded; there is impaired viability and reduced fertility due to disruption of spermatogenesis (Kress et al., 2007). CELF3 knock-out mice did not show any reduction in fertility but similar to CELF1 knock-out mice, they have reduced spermatogenesis (Dev et al., 2007). This may be the result of the compensatory effect of other members of the CELF family. The expression of CELF3 is restricted to the mouse brain and testis although it is exclusively expressed in human brain (Dev et al., 2007). CELF4 knock-out mice have similar phenotype as the frequent flyer mouse model, whose *celf4* gene is disrupted resulting in a complex seizure phenotype (Yang et al., 2007). CELF4 regulates the expression of genes essential for neuronal viability, which are subsequently down-regulated in mutant mice, two (Htr2c and Syn2) that cause seizures when knocked-out in mice (Yang et al., 2007). Hence, disruption of the *CELF4* gene results in a disease phenotype probably due to its impaired function.

1.7 RNA binding proteins in disease

Mutations that disrupt alternative splicing can lead to cellular dysfunction and disease as explained with FTDP-17 mutations. Furthermore, misregulation of the activity of RNA-binding proteins can also result in aberrant splicing of their target pre-mRNA transcripts. The regulation of alternative splicing is complex, depending on loosely conserved *cis*-acting regulatory sequence elements, *trans*-acting protein factors as well as cellular responses dependent on environmental conditions. The following sections describe the role of misregulated RNA-binding proteins in neurological disorders.

Several *trans*-acting factors/splicing factors interact with regulatory elements in the *MAPT* gene during tau exon 10 splicing as explained above. They include members of the SR proteins family and CELF family of RNA-binding proteins (Andreadis, 2005).

1.7.1 SR proteins in disease

The identification of SR proteins as essential factors in the control of alternative splicing and cell growth make them potential candidates for specific disease conditions when they are misregulated either through post-translational modifications or expression. SR proteins exhibit their function in disease in several ways as follows.

1.7.1.1 Gene transcripts regulated by SR proteins are implicated in disease

SR proteins regulate some gene transcripts implicated in disease; these include oncogens and tumour suppressor genes. A typical example of this is that ASF regulates the alternative splicing of *Ron* proto-oncogene (a tyrosine kinase receptor for a macrophage protein which stimulates proliferation while inhibiting apoptosis). A mutant auto-activating form of *Ron* confers high metastatic potential leading to production of a protein product that is directly involved in the invasive characteristic of tumour cells. Since SR proteins exert their function in a dose dependent manner (Karni et al., 2007), it is conceivable that altering their expression may cause noticeable changes in activity. For instance, cellular deficits may occur during proliferation and differentiation (Karni et al., 2007). Indeed, altering the alternative splicing pattern of target genes (such as *CD44* gene), by SR proteins has been reported in ovarian and breast cancers where Tra2β and SRp55 were increased in primary tumours (Fischer et al., 2004).

1.7.1.2 Recognition of point and deletion mutations by SR proteins

SR proteins have the ability to recognise point and deletion mutations in disease-causing genes. For example, in spinal muscular atrophy (SMA), aberrant alternative splicing results in disease phenotype. SMA is a recessive disorder in humans and a prevalent cause of infant mortality. It is a form of motor neuron disease caused by deletion mutations of a functional *SMN1* gene that encodes for the survival motor neurone (SMN) protein. Ubiquitously expressed SMN is a

splicing factor required for the synthesis and assembly of snRNP to form the SMN complex (Gubitz et al., 2004). SMN associates with mRNA binding proteins, such as HuD and transports poly (A) mRNA, in the axons of motor neurons (Fallini et al., 2011). A loss of SMN function results in neurodegeneration of motor neurons especially common in the spinal cord.

SMA is considered a general splicing disease. Loss of function of SMN1 cannot be compensated for by its almost identical gene, *SMN2*. *SMN2* has a silent C > T substitution in the sixth nucleotide of exon 7 which results in exon 7 skipping and the production of a non-functional, truncated SMN protein, SMN2 (Coovert et al., 1997; Le et al., 2005; Monani, 2005). This SMN2 mutation causes a loss of an ESE by abolishing the binding site for the SR protein, SF2/ASF and creation of an ESS to which the hnRNP A1 binds (Cartegni et al., 2002). Furthermore, the SR-related protein, Tra2β promotes SMN2 exon 7 inclusion. Therefore, modulation of Tra2β may be an avenue to therapeutically correct aberrant SMN2 splicing (Hofmann et al., 2000).

1.7.1.3 Phosphorylation of SR proteins is misregulation in tauopathies

Glycogen synthase kinase 3 (GSK-3β) is one of the kinases that phosphorylates tau protein *in vitro* to an extent where its characteristics resembles PHF-tau in AD (Hanger et al., 1992; Lovestone et al., 1994). GSK-3β inhibition in rat cortical neurons causes an increase in the expression of the SR protein, SC35 in nuclear speckles, which leads to an increase in tau exon 10 inclusion (Hernandez et al., 2004). Hence, misregulation of GSK-3β activity may alter tau exon 10 splicing through SC35 phosphorylation-dependent changes in localisation and activity, which may contribute to the aggregation of tau in NFTs observed in AD.

Another kinase that phosphorylates the SR protein, ASF, is Dyrk1a kinase which has been implicated in Down's syndrome (DS) tauopathy. There is an extra copy of chromosome 21 in DS patients as well as early onset of AD like tau pathology (Wisniewski et al., 1985a; Wisniewski et al., 1985b). ASF plays a very important

role in promoting tau exon 10 inclusion. Dyrk1a kinase phosphorylates ASF and regulates its role in tau splicing (Shi et al., 2008). Phosphorylation of ASF by Dyrk1a results in its localisation into nuclear speckles. This prevents ASF promotion of tau exon 10 inclusion causing an increase in tau transcripts with only three microtubule binding repeats (3R tau) and may contribute to early onset of tauopathy in DS patients (Shi et al., 2008).

1.7.1.4 Misregulation of SR proteins during splicing in tauopathies

Several SR proteins have been implicated in the regulation of tau alternative splicing including Tra2β which activates tau exon 10 splicing by binding to a purine-rich enhance (PPE) element in exon 10 (Kondo et al., 2004; Wang et al., 2005). The expression levels of Tra2β (therefore its activity) was significantly increased in the temporal cortex of AD cases (Glatz et al., 2006).

1.7.2 Molecular pathology of myotonic dystrophy (DM)

Disruption of alternative splicing regulation leads to diseases such as myotonic dystrophy (DM). DM1 is an autosomal dominant disease characterised by delayed relaxation of muscle contractions (myotonia), progressive myopathy, cardiac conduction abnormalities and cataracts (Ladd et al., 2001). In DM, pathogenic mutations occur due to unstable repeat expansion which leads to altered RNA function and consequently, the increased expression of non-coding and non-translated repeats. In DM, there is an expansion of the CUG or CCUG repeats in the 3'-UTR of dystrophia myotonica protein kinase (*DMPK*) mRNA and in intron 1 of *ZNF9* pre-mRNA resulting in DM1 and DM2 respectively (Fuger et al., 1995; Ho et al., 2005; Mahadevan et al., 1992). Pathogenesis of DM is due to altered alternative splicing caused by the sequestration of RNA-binding proteins (such as muscleblind, MBNL1) which results in abnormal splicing regulation of their pre-mRNA transcripts which may include tau (Sergeant et al., 2001).

1.7.2.1 Tau pathology in myotonic dystrophy (DM)

Although DM1 is a muscle wasting disease, elderly patients show signs of neurodegeneration (de Leon and Cisneros, 2008). Neurodegeneration in DM1 is noticeable as AD-like aggregated tau pathology in the cortical areas including the hippocampus, entorhinal cortex and temporal areas of aged patients similar to that observed in AD (Sergeant et al., 2001; Vermersch et al., 1996). In the cortical neurons of DM1 patients, a decrease in tau isoforms containing exon 2, 3 and 10 was detected (Jiang et al., 2004; Sergeant et al., 2001; Vermersch et al., 1996). CELF3 and CELF4 promote the inclusion of tau exon 10 *in vitro* (Andreadis, 2005; Wang et al., 2004). It is likely that deficient CELF protein activity in DM1 brain for example, CELF4 (Leroy et al., 2006) may contribute to the expression of tau transcripts without exon 10 (Andreadis, 2005; Wang et al., 2004).

1.7.2.2 CELF proteins in myotonic dystrophy

In the brain of DM1 patients, CELF protein expression is misregulated and thus, their activity is altered. A prominent decrease in the expression of CELF1, CELF2 and CELF4 transcripts was observed at RNA level in DM1 brain although CELF1 is up-regulated in the skeletal muscle of DM1 patients (Leroy et al., 2006). Hence, these deficiencies of CELF protein expression at RNA level in DM1 brain may contribute to the decrease of tau exon 10 transcripts (Dhaenens et al., 2011).

The important role of CELF1 in the pathogenesis of DM1 was highlighted by the altered splicing of its pre-mRNA targets. CELF1 has a high affinity for CUG or CCUG repeats (Ladd et al., 2001) (Douglas and Wood, 2011). DMPK transcripts containing expanded CUG repeats are aggregated in the 3’ UTR of the pre-mRNA causes a gain of function mutation of CELF1. The expanded CUG repeats increases the activity of PKC for which CELF1 is a downstream target. Increased phosphorylation of CELF1 stabilises it, resulting in up-regulation of its activity (Kuyumcu-Martinez et al., 2007). This causes abnormal splicing of CELF1 downstream transcripts seen to be abnormally expressed in DM1 patients (Philips et al., 1998; Ranum and Cooper, 2006). For example, the insulin receptor (IR)

showed increased exon 11 exclusion which causes insulin resistance (Savkur et al., 2001) while increase in cardiac troponin T (cTNT) exon 5 inclusion results in cardiac muscle defects. In addition, muscle-specific chloride channel gene (*CLNC-1*) had increased inclusion of intron 2 as well as two pseudo exons between exons 6 and 7. This produced premature stop codons which cause non-sense mediated decay of *CLNC-1* transcripts and reduced expression of *CLNC-1* and abnormal chloride conductance in muscle causing myotonia (Charlet et al., 2002).

1.7.3 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a progressive adult-onset neurodegenerative disorder characterised by the loss of motor neurons in the brain and spinal cord resulting in cell death and progressive muscle weakness (Shaw, 2001). Approximately 10 % of all ALS cases are familial (FALS) while the rest are sporadic (SALS). 20 % of these FALS are due to mutations in Cu/Zn superoxide dismutase 1 (*SOD1*) and 1 % of all sporadic (SALS) cases (Pasinelli and Brown, 2006; Rosen, 1993). Another mutation that causes ALS is in the RNA/DNA-binding FUS/TLS (fused in sarcoma/translocated in liposarcoma) gene which causes some familial ALS (Kwiatkowski et al., 2009; Vance et al., 2009).

ALS is characterised by the presence of polyubiquitinated inclusions (UBIs) in the soma and proximal axon of surviving motor neurons (Leigh et al., 1991). Furthermore, FTLD-U (frontotemporal lobar degeneration with ubiquitin-positive inclusions), a tau negative and ubiquitin positive frontotemporal lobar dementia also have pathological UBIs in the cortical neurons of both the frontal and temporal lobes of patients (Lipton et al., 2004).

The identification of TDP-43 (see below) as a major constituent of both ALS and FTLD-U led to them been grouped as ‘TDP-43 proteinopathies’ because of their similar clinico-pathological phenotype (Arai et al., 2006; Cairns et al., 2007; Neumann et al., 2006).

1.7.3.1 TDP-43 in ALS

TDP-43 (TAR DNA binding protein 43) is a DNA and RNA binding protein implicated in alternative splicing regulation and mRNA transport. TDP-43 was identified through a search for proteins binding UG repeat regions (Buratti et al., 2001). TDP-43 binds to UG repeats in intron 8 of CFTR pre-mRNA and regulates of exon 9 splicing (promotes exon 9 skipping) (Buratti et al., 2001). TDP-43 mediates splicing activity through its glycine-rich C-terminal, necessary for CFTR exon 9 splicing (Buratti and Baralle, 2008). Structurally, TDP-43 is similar to members of the hnRNP family, with two RRMs flanked by N- and C-terminal domains (Figure 1.9). RRM1 is implicated in the RNA and DNA binding properties of the protein (Buratti and Baralle, 2008; Ou et al., 1995).

TDP-43 disease associated mutations were recognized by several groups in a subset of FALS cases and some SALS cases through direct sequencing (Gitcho et al., 2008; Kabashi et al., 2008; Kuhnlein et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008). The glycine-rich C-terminal of TDP-43 harbours majority of the disease-related mutations (accountable for protein-protein interactions with members of the hnRNP family) except for D169G found within RRM1 (Buratti et al., 2001). Most of these mutations are missense mutations except for Y374X, which causes C-terminal truncation (Daoud et al., 2009). The disturbance of TDP-43 interaction with other splicing factors, such as hnRNP A2 does not alter protein function, but may contribute to pathogenesis of disease through aggregate formation (D'Ambrogio et al., 2009).

In ALS and FTDL-U, TDP-43, is mislocalised to the cytoplasm from the nucleus where it is ubiquitinated, hyperphosphorylated, and cleaved, producing C-terminal fragments (Arai et al., 2006; Neumann et al., 2006; Sreedharan et al., 2008). The TDP-43 pathology in these diseases coincides with cytoplasmic aggregation (Kwong et al., 2007; Van Deerlin et al., 2008). This may occur due to combination of toxic gain of function and loss of normal activity of TDP-43.

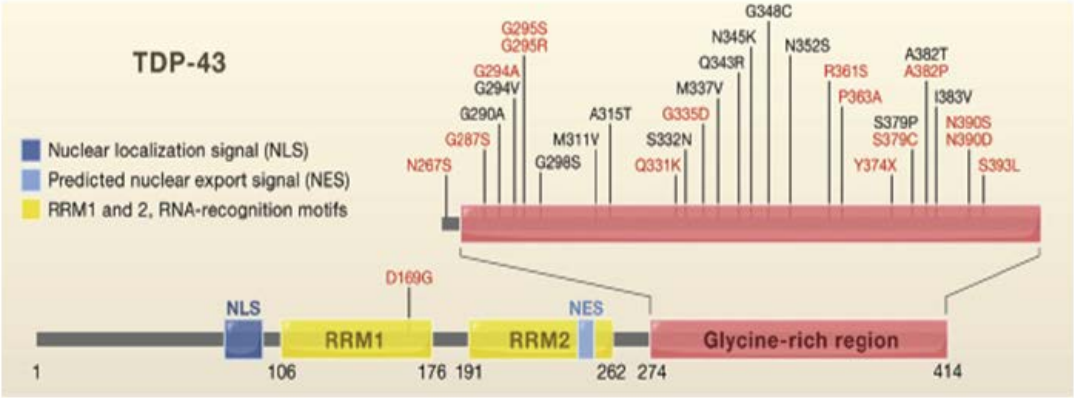


Figure 1.9. Structural domains and disease causing mutations of TDP-43 (from (Lagier-Tourenne and Cleveland, 2009)).

TDP-43 is an RNA and DNA binding protein with two conserved RRMs and a well defined C terminal glycine rich domain. The majority of disease-causing mutations are found within the C-terminal domain with the exception of D169G located in RRM1.

The loss of TDP-43 from the nucleus implies that its functional properties in the nucleus during RNA metabolism are lost and may contribute to pathology (Lagier-Tourenne et al., 2010; Neumann et al., 2006; Sreedharan et al., 2008). The depletion of TDP-43 using antisense oligonucleotides in adult mouse brain revealed a decrease in the expression levels of about 1000 RNAs with very long introns having binding sites for TDP-43 (Polymenidou et al., 2011). The expression of the long mRNAs in the brain recognises their neuronal susceptibility to TDP-43 depletion (Polymenidou et al., 2011). Furthermore, iCLIP (individual nucleotide-recognition cross-linking immunoprecipitation) was used to identify the relationship between TDP-43 and long intronic regions containing UG-rich sequences *in vivo* (Tollervey et al., 2011). RNA-protein interactions of TDP-43 were identified in FTLD patients having characteristic TDP-43 inclusions. FTLD patients showed strong interaction between TDP-43 and small nucleolar RNAs, small nuclear RNAs as well as ribosomal RNAs (Tollervey et al., 2011). In addition, knockdown of TDP-43 in neuroblastoma cells revealed that 158 alternative exons were down-regulated including genes involved in neuronal development (Tollervey et al., 2011).

TDP-43 positive inclusions have been increasingly recognised in other neurodegenerative conditions, referred to as secondary TDP-43 proteinopathies. A prominent member of this is AD with TDP-43 pathology (Amador-Ortiz et al., 2007b).

1.7.3.2 TDP-43 in AD

TDP-43 inclusions frequently accompany the pathological hallmarks observed in AD (Amador-Ortiz et al., 2007b; Josephs et al., 2008; Uryu et al., 2008). These TDP-43 inclusions are present in the neurons and glia of roughly 20 – 30 % of sporadic AD cases (Amador-Ortiz et al., 2007b; Uryu et al., 2008) and about 14 % in familial AD (Lippa et al., 2009). However, TDP-43 pathology in AD is restricted to the limbic regions of the brain including the hippocampus, amygdala and surrounding cortices (Figure 1.10) (Amador-Ortiz et al., 2007a; Uryu et al., 2008).

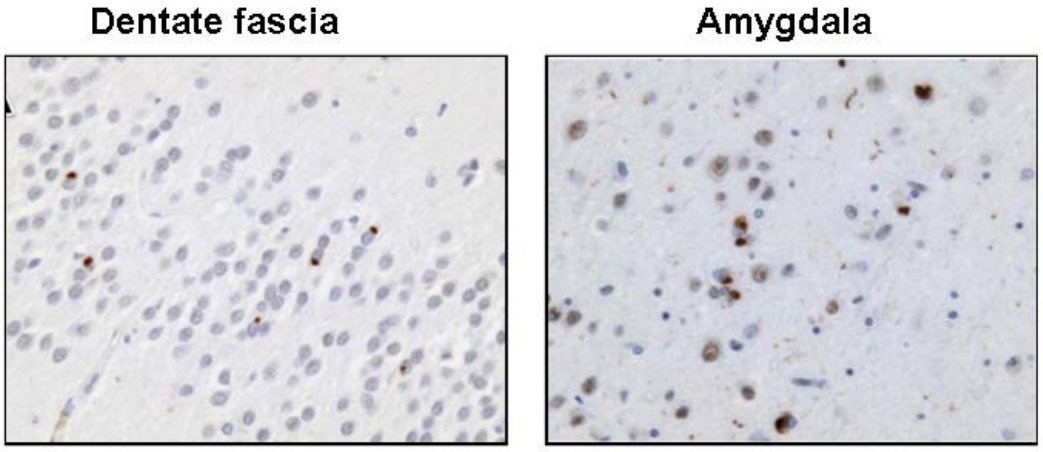


Figure 1.10. TDP-43 inclusions in AD with hippocampal sclerosis (from (Amador-Ortiz et al., 2007b)).

AD case with hippocampal sclerosis showing TDP-43 positive cytoplasmic inclusions in the dentate fascia (part of the hippocampal circuit) and the amygdala.

Moreover, TDP-43 pathology in AD shows an overlap with tau pathology distribution (neuropil threads and NFTs) (Fujishiro et al., 2009) which bears resemblance with those in FTLD-TDP including the presence of neuronal cytoplasmic and intranuclear inclusions; particularly in neurons susceptible to NFTs. Immunochemical analysis of AD brain using specific antibodies to pathogenic forms of TDP-43 including C-terminal epitopes and phospho-epitopes were correlated with the presence of abnormal TDP-43 (Arai et al., 2009).

These studies propose that TDP-43 inclusions in AD cases may contribute to pathogenesis of disease associated with greater brain atrophy principally in the hippocampus as well as severe clinical phenotype (Josephs et al., 2008). This is in agreement with the finding that hippocampal atrophy present in AD cases with TDP-43 pathology is more prominent than in AD cases without TDP-43 pathology (Josephs et al., 2008).

1.7.3.3 TDP-43 and its interacting RNA binding proteins

Several RNA-binding proteins that regulate RNA metabolism show extensive interaction with TDP-43 (Freibaum et al., 2010; Ling et al., 2010). Some of the splicing factors interacting with TDP-43 depend on the N-terminal RRM1 to mediate RNA-protein functions of TDP-43 (Freibaum et al., 2010). The interactions of TDP-43 with splicing factors are in two distinct clusters:

- **A nuclear/splicing cluster**. A group of nuclear proteins that control RNA splicing as well as other nuclear RNA metabolism were found in this cluster. They include many hnRNPs, SR proteins, snRNPs, and nuclear RNA export factors. These proteins are all implicated in nuclear RNA metabolism (mainly RNA splicing and mRNA export to the cytoplasm) (Freibaum et al., 2010; Ling et al., 2010).

- **A cytoplasmic/translation cluster**. This includes a group of cytoplasmic proteins that regulate mRNA translation. Members of this cluster include translation initiation and elongation factors (Freibaum et al., 2010).

This is in accordance with the suggestion that TDP-43 has numerous functions in RNA metabolism in the nucleus and cytoplasm. Proteins interacting with TDP-43 include some members of CELF proteins and SR proteins family (Freibaum et al., 2010 660). These splicing factors may be under the influence of TDP-43 to mediate activities other than splicing such as control of mRNA export, localisation and stabilisation.

Two members of the SR family of proteins were found in the nuclear splicing cluster of TDP-43 including 9G8 and SRp55 (Freibaum et al., 2010). However, the only CELF protein identified as an interactor of TDP-43 is CELF1 (Ling et al., 2010); (Freibaum et al., 2010). TDP-43 (like CELF2) regulates CFTR exon 9 skipping by binding an ISE motif at the end of intron 8 (Dujardin et al., 2010). Whilst CELF1 binds to this same ISE motif, it does not promote CFTR exon 9 skipping (Dujardin et al., 2010).

There are potential similarities between DM1 and TDP-43 proteinopathies. In TDP-43 proteinopathies, TDP-43 is sequestered into cytoplasmic aggregates in neurons while in DM1, there is gain of function of splicing factors (CELF1) in the nucleus. In both diseases, the splicing pattern of their downstream pre-mRNA targets is altered. Although, the exact relationship between mis-splicing of mRNA targets and neurodegeneration in DM1 is not known; it is plausible that in TDP-43 proteinopathies (such as AD with TDP-43 pathology), RNA-binding proteins may be sequestered into aggregates preventing their splicing activity.

1.8 Drugs targeting SR rich proteins

The inhibition of splicing regulators either directly or indirectly by small chemicals could correct abnormal splicing (Douglas and Wood, 2011; Soret et al., 2005). Splicing factors that rely entirely on the use of ESE sequences can be specifically inhibited to correct abnormal splicing. These drugs show highly selective inhibition of splicing processes mediated by ESE sequences (Soret et al., 2005). For example the C77 and C83 drugs inhibit the activity of members of the SR family that are essential for spliceosome assembly through direct interaction with them (Soret et al., 2005).

1.8.1 Kinases as a therapeutic target

In terms of alternative splicing of pre-mRNAs, the phosphorylation state of splicing factors may determine if an exon is skipped or included in mRNA. The phosphorylation state of splicing factors has been implicated in AD where regulators of tau exon 10 alternative splicing were altered (Glatz et al., 2006). Analysis of the splicing patterns of exon 10 in the temporal cortex of sporadic AD brain showed decrease in Clk2 activity (a kinase, that phosphorylates tra2β1), increase human tra2β-1 mRNA, and increased exon 10 containing tau mRNA (Glatz et al., 2006). However, a decreased expression of htra2β-1 protein was reported in sporadic AD cases (Conrad et al., 2007). This suggests that defects in pre-mRNA processing contribute to sporadic AD and altering the phosphorylation state of splicing factors may be beneficial. However, modulation of the phosphorylation state of splicing factors in therapy may propose an obstacle because kinases or phosphatases have more than one downstream target thereby modulating SR-mediated splicing events. Therefore, altering the activity or expression of an enzyme may not have just the desired local effects but exert their effect on other cellular mechanisms apart from splicing.

1.8.2 Potential of gene therapy

Generation of therapies that can reverse or restore mis-regulated splicing events linked to several human genetic disorders is essential for correcting abnormal splicing. To correct abnormal alternative splicing, direct intervention at the RNA level is required. Mutated sequences in regions of a gene such as splice sites or regulatory sequences that cause the production of dysfunctional proteins are targets for techniques used to correct aberrant splicing.

A more efficient approach compared to altering the phosphorylation state of splicing factors may be the viral delivery of splicing factors to promote exon skipping or inclusion.

1.8.2.1 Antisense oligonucleotides

Antisense oligonucleotides modulate splicing in a target-specific manner. Oligonucleotides that are complementary to a specific RNA sequence can be synthesised to target splice sites, enhancer or silence elements within a RNA transcript (Douglas and Wood, 2011). This allows manipulation of the splicing machinery in a precise manner. Oligonucleotides binding to tau exon 10 splice junctions have been used to decrease the expression of exon 10 in PC12 cells (Kalbfuss et al., 2001). This application of antisense oligonucleotides directed against tau exon 10 splice junctions suppresses inclusion of tau exon 10 by forming a stable pre-mRNA-oligonucleotide hybrid, which blocks access of the splicing machinery to the pre-mRNA. The advantage of this technique is that the modified transcript's expression pattern is the same as the endogenous transcript. This is because the modified RNA transcript is expressed under the influence of the gene's transcriptional control (Douglas and Wood, 2011). Furthermore, the synthesis of antisense oligonucleotides that binds to CUG repeats in DM1 will prevent the binding and sequestration of splicing factors such as MBNL1 to counter the loss of function that accounts of DM1 pathology.

1.8.2.2 RNA interference (RNAi)

Eukaryotes modulate gene expression by RNAi at pre- and post-transcriptional levels (Gaur, 2006). RNAi mediated gene silencing is induced by small interference RNA (siRNA) produced from small hairpin RNA (shRNA) by Pol III promoter and transfer RNA (tRNA) promoters (Gaur, 2006). After transcription, intrinsic RNAse III processes shRNAs into siRNA duplexes followed by incorporation of the siRNA strand into the RNA-induced silencing complex (RISC). siRNA selectivity is paramount in silencing disease causing mRNA without affecting the levels of wild-type allele or an isoform that has a vital function (Gaur, 2006).

1.8.2.3 Correcting abnormal alternative splicing using SMART™

With SMART™, a chimaeric mRNA is created by trans-splicing where the spliceosome catalyses the reaction between the 5' splice site of a natural target pre-mRNA and the 3' splice site of an exogenous delivered pre-*trans*-splicing molecules (PTM) RNA molecule (Gallo et al., 2007; Puttaraju et al., 1999). The reprogramming of tau pre-mRNA using spliceosome-mediated RNA trans-splicing (SMART™) can correct aberrant alternative splicing. SMART™ corrects abnormal splicing at the RNA level such that the correct transcript is expressed and the mutant form of the transcript is down-regulated concurrently (Gallo et al., 2007). SMART adequately modulates exon 10 splicing of tau pre-mRNA. Using SMART™, exclusion of exon 10 was achieved with 30 % efficiency (Rodriguez-Martin et al., 2005) (Rodriguez-Martin et al., 2009). In the context of tauopathies with increased expression of tau exon 10 mRNA such as some FTDP-17 mutations, SMART™ can be used to create a chimeric mature transcript to correct abnormal tau isoform ratio (Rodriguez-Martin et al., 2005). Treatment of diseases caused as a result of defective alternative splicing by directly interfering with the RNA requires the delivery of the PTM through viral vectors. This avoids the obstacles of the blood brain barrier as well as decreasing the possibility of side-effects (Gallo et al., 2007).

1.9 Summary

The dynamic regulation of alternative splicing by a stringent combination of *trans*-acting factors and *cis*-acting elements is important for the proper functioning of neurons. Misregulation of alternative splicing can result in diseases due to alteration in the levels of mature transcripts and therefore protein isoforms as explained in this Chapter. This review has highlighted the important roles that RNA-binding proteins particularly, SR proteins and CELF proteins play in RNA processing, and suggests that they may contribute to pathogenesis of sporadic AD. This could occur due to misregulation of the activity of these RNA-binding proteins that govern the activity of *cis*-acting elements during tau exon 10 alternative splicing. The misregulation of RNA-binding proteins such as SR proteins and CELF proteins in neurodegenerative disease makes them potential targets of therapeutic intervention in the treatment of these diseases. Direct modulation of splicing factors through their expression and/or phosphorylation state provides a means by which their splicing activity can be manipulated with drugs to correct aberrant alternative splicing events. For instance, modulating the phosphorylation state of the SR protein, SC35 (which promotes tau exon 10 inclusion) through the activity of the kinase, GSK-3β (Hernandez et al., 2004) using drugs that directly inhibit it may provide therapeutic benefit. Furthermore, manipulating the machinery that controls splicing to repair misregulated alternative splicing provides another avenue for therapeutic intervention.

1.10 Aim of thesis

The rationale of this study is that the activity of splicing factors that associate with tau pre-mRNA transcript during RNA processing may be misregulated in sporadic tauopathies (for example, changes in expression level), which could lead to their identification as novel therapeutic targets. Potential targets include CELF1 and CELF2 implicated in the pathogenesis of DM1; as well as CELF3 and CELF4, implicated in tau alternative splicing. Also, SC35, SRp40 and SRp55 are modulators of tau alternative splicing.

The specific aim of this project is:

To investigate whether expression of splicing factors that modulate tau splicing is correlated with tau exon 10 splicing in brain regions affected in AD.

The specific objectives of this thesis are:

- To determine total levels of specific CELF proteins by quantitative western blotting in brain regions affected in AD (temporal cortex, amygdala and hippocampus) using human post-mortem brain tissue from AD patients (with (+) and without (-) TDP-43 inclusions) and age-matched controls.

- To determine, the levels of transcripts encoding specific CELF proteins (CELF1, CELF3 and CELF4) and SR proteins (SRp55, SRp40 and SC35) using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Affected and unaffected areas from human post-mortem brain tissue of AD patients (with (+) and without (-) TDP-43 inclusions) and age-matched controls were analysed.

- To correlate the expression levels of CELF proteins and SR proteins with tau exon 10 splicing in the same AD cases and age-matched controls.

The comparison of the levels of expression of the splicing factors in normal and diseased brain will give an insight to whether the expression of splicing factors changes in sporadic AD. This will elucidate their importance in neurodegeneration. Understanding the mechanisms by which splicing factors regulate alternative splicing of tau in normal and diseased human brain will provide another avenue with which to therapeutically intervene in these dementias.

Chapter Two

2 Materials and methods

2.1 Materials

Molecular biology, microbiology and cell culture reagents were purchased from Invitrogen Ltd. (Paisley, UK), unless otherwise stated. All other chemicals were purchased from Sigma-Aldrich Company limited (Dorset, UK) except where indicated. Ultrapure water from an Elga Maxima water purification system was used to prepare stock and buffer solutions.

2.1.1 General reagents and stock solutions

- Albumin, bovine (1mg/ml), BSA in ultra-pure water)
- Agarose
- Ammonium persulphate (APS), 10 % (w/v) (National Diagnostics Ltd., Yorkshire, UK)
- Ampicillin, 100 mg/ml in ultra-pure water
- Bromophenol blue, 0.5 % (w/v)
- Dithiothreitol (DTT)
- Ethanol, ~99.9 % (v/v) (Merck Biosciences, Hertfordshire, UK)
- Ethidium bromide (10 mg/ml)
- Ethylenediaminetetra-acetic acid (EDTA) (Merck Biosciences, Hertfordshire, UK)
- Foetal bovine serum (FBS) (Sera Laboratories International, West Sussex, UK)
- Hoechst 33258
- Isopropyl-beta-D-thiogalactopyranoside (IPTG)
- L-glutamine
- Glycerol ~98 % (v/v)
- Isopropanol (Merck Biosciences, Hertfordshire, UK)
- Beta-mercaptoethanol (Merck Biosciences, Hertfordshire, UK)

- Methanol, 100 % (v/v) (Merck Biosciences, Hertfordshire, UK)
- Orange G
- Penicillin/ Streptomycin
- Polyoxyethylene sorbitan monolaurate (Tween 20)
- Sodium dodecyl sulphate (SDS) (National Diagnostics Ltd.)
- SYBR Green I (Roche Diagnostics)
- N,N,N',N'-tetra-methylethylenediamine(TEMED)(National Diagnostics Ltd.)
- TRIS (hydroxymethyl) aminomethane acetate salt
- TRIS (hydroxymethyl) aminomethane hydrochloride
- Triton X-100, 10 % (v/v)
- Trizol® reagent (Invitrogen)
- Trypsin-EDTA
- X-gal (50mg/ml) (Promega)

2.1.2 Bacterial strains

Escherichia coli (E.coli) host strain, JM109 (Promega, Southampton, UK) was used to amplify plasmids.

2.1.3 Bacteria culture media and reagents

2.1.3.1 Luria-Bertani (LB) broth base

A pre-mixed powder consisting of (per one litre): 1 % (w/v) SELECT Peptone 140, 0.5 % (w/v) NaCl and 0.5 % (w/v) SELECT yeast extract, pH 7.0 was purchased. A 2 % (w/v) solution was prepared in 1 litre culture flasks and autoclaved at 121°C for 15 min. To achieve adequate aeration, a maximum volume of 200 ml LB was used per flask.

2.1.3.2 LB ampicillin (LB-amp) medium

A Nalgene syringe filter, 0.2 μm pore size was used to prepare filter-sterilised stock solution of ampicillin, 100 mg/ml in ultra-pure water. A final concentration of 100 μg/ml ampicillin was added to, autoclaved and cooled LB.

2.1.3.3 LB-agar

A pre-mixed powder consisting of 1.2 % (w/v) SELECT-agar in LB was purchased. A 3.2 % (w/v) solution of LB-agar was prepared in ultra-pure water and autoclaved at 121°C for 15 min prior to use.

2.1.3.4 LB-amp agar

Ampicillin stock solution was added to cooled, autoclaved LB-agar to give a final concentration of 100 μg/ml. LB-amp agar was poured into 10 cm diameter sterile Petri dishes two-thirds to the top and allowed to cool.

2.1.4 Solutions for the preparation of plasmid DNA

2.1.4.1 Miniprep

All solutions were purchased as part of a QIAprep® Spin Miniprep Kit (Qiagen, West Sussex, UK).

2.1.5 DNA analysis solutions

2.1.5.1 Tris-acetate-EDTA (TAE) 50×

2M Tris-acetate (pH 8.0)
50 mM EDTA

2.1.5.2 Agarose gel loading solution 6×

0.25 % (w/v) orange G

40 % (w/v) sucrose

2.1.5.3 DNA size markers

Quick-load™ 1kb DNA ladder (New England BioLabs, Hertfordshire, UK): 10002, 8001, 6001, 5001, 4001, 3001, 2000, 1500, 1000, 517 and 500 bp fragments. The 3001 bp fragment is of a higher concentration for easy identification.

Quick-load™ 100 bp DNA ladder (New England BioLabs): 1517, 1200, 1000, 900, 800, 700, 600, 500, 517, 400, 300, 200, 100 bp fragments. The 1000 and 500 bp fragments are of a higher concentration for easy identification.

2.1.5.4 QIAquick gel extraction/reaction cleanup kit

QIAquick kit (Qiagen) was used to extract and purify DNA from agarose gels.

2.1.6 RNA analysis solutions

2.1.6.1 RNA extraction

RNA was extracted from cells using Trizol® reagent, a solution of phenol and guanidine isothiocyanate.

2.1.7 Reverse transcription solutions

Reverse transcription (RT) was performed using a kit (Applied Biosystems, Cheshire, UK). The reagents are listed as final concentrations with stock concentrations in brackets. Non-DEPC treated, nuclease free water was purchased from Ambion Ltd. (Cheshire, UK).

- 1× RT buffer (10× RT buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3)
- 55 mM magnesium chloride (25 mM)
- 500 μM of each deoxyNTPs (dNTPs) (dNTP mixture containing 2.5 mM of dATP, dCTP, dGTP and dTTP)
- 25 μM oligo-dT (dT16) (50 μM in 10 mM Tris-HCl pH 8.3)
- 0.4 U/μl RNase inhibitor (20 U/μl in 20 mM HEPES-KOH, pH 7.6, 50 mM KCl, 8 mM DTT, 50 % (v/v) glycerol)
- 1.25 U/μl MultiScribe Reverse Transcriptase (50 U/μl)

2.1.8 PCR Solutions

2.3.1.1 Taq DNA polymerase in storage buffer B

PCR was carried out using Taq DNA polymerase in storage buffer B (Promega). Taq DNA polymerase is a thermostable enzyme that replicates DNA at 74 ºC and exhibits a half-life of 40 min at 95 ºC. Taq catalyses the polymerisation of nucleotides into duplex DNA in the 5'-3' direction in the presence of magnesium.

2.3.1.2 Taq DNA polymerase 5× buffer

Containing (working concentration 1:10)

- 50 mM KCl
- 10 mM Tris-HCl pH 9.0
- 1.5 mM MgCl2
- % (v/v) Triton X-100

2.3.1.3 2'-Deoxynucleoside 5'-triphosphates (dNTPs)

Each nucleotide (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) was supplied as a 100 mM solution in sterile ultra-pure water (pH 7.5). A 12.5× stock solution containing 2.5 mM of each dNTP was prepared by mixing 5 µl of dATP, dCTP, dGTP and dTTP with 180 µl of sterile ddH_20.

2.3.1.4 Primers

Primers were synthesised by MWG-Biotech and diluted to a working stock solution of 2 µM. The melting temperature (Tm) is defined as the temperature at which half of the strands are in the double-helical state and half are in the "random-coil" state. Different formulas for calculating Tm have been described in the literature; consequently, Tm varies according to the formula chosen for their calculation. In the present work, Tm were calculated by MWG-Biotech and provided with each oligonucleotide synthesis report (Tm = 69.3 + 0.41 x GC % - 650/length of sequence). The primers used in this work are described in Table 2.1.

2.3.1.5 Quantitative reverse transcription polymerase chain reaction

FastStart SYBR Green Master (Rox) 2×

Table 2.1. **Primers used**

	Name	Location	Sequence	Product(s) Size (bp)	
GAPDH	G1	Exon 4	5'-GCCCAATACGACCAA ATCC-3'	66	
	G2	Exon6	5'-AGCCACATCGCTCAGACAC-3'		
β-actin	βA1	Exon 5	5'-CCAGAGGCGTACAGG GATAG-3'	97	
	βA2	Exon 6	5'-CCAACCGCGAGAAGATGA-3'		
Tau 9-13 (Figure 2.1)	E9	Exon 9	5'- IR700-CTGAAGCACCAGCCAGGAGG-3'	E10+	367
	E13	Exon 13	5'-TGGTCTGTCTTGGCTTTGGC-3'	E10-	274
Total Tau	TF	Exon 8	5'- GATTGGGTCCCTGGACAATA -3'	106	
	TR	Exon 9	5'- GTGGTCTGTCTTGGCTTTGG -3'		
SC35	35F	Exon 3	5'-CTACAGCCGCTCGAAGTCTC -3'	171	
	35R	Exon 3	5'- GATTCCCTCTTGGACACTGG-3'		
SRp40	40F	Exon 6	5'-GGAAGTAACGTTTGCGGATG-3'	177	
	40R	Exon 8	5'- GGATCGAGACCTGCTTCTTG -3'		
SRp55	55F	Exon 4	5'-CACAAGCCATAGGCGATCTT-3'	172	
	55R	Exon 6	5'- CCTGCCTTTTGATCGAGAAC-3'		
CELF1	C1F	Exon 8	5'-TCCTCTGGAACCTCAACAC-3'	236	
	C1R	Exon 10	5'-AGGGCCCAAGTGAGGCTAT-3'		
CELF3	C3F	Exon 8	5'-CTGCCCCTGATGCTCTGTAT-3'	209	
	C3R	Exon 10	5'-CTGCTGCTGTTGAGGTGG-3'		
CELF4	C4F	Exon 7	5'-CACCTATGACCCCAACCTCA-3'	236	
	C4R	Exon 9	ACCTGCATACTGCTGCACTC -3'		

2.3.2 Nuclease free water

2.3.3 TURBO DNase (2 U/µl)

- pTRI-Xef, 0.5 mg/ml (Control Template)
- Ammonium acetate stop solution, 5 M ammonium acetate, 100 mM EDTA
- Lithium chloride precipitation solution, 7.5 M lithium chloride, 50 mM EDTA
- 10× Reaction buffer
- ATP solution, 75 mM
- CTP solution, 75 mM
- GTP solution, 75 mM

2.3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) solutions

2.1.11.1 Laemmli sample buffer (2×)

- 125 mM TRIS-HCl pH 6.8
- 4 % (w/v) SDS
- 5 % (v/v) β-mercaptoethanol
- 20 % (v/v) glycerol
- 0.05 % (v/v) bromophenol blue

2.1.11.2 Acrylamide working stock

Acrylamide stock was purchased as ProtoGel™ solution (National Diagnostics Ltd.) and consists of:

- 30 % (w/v) acrylamide
- 0.8 % (w/v) bis-acrylamide (37.5:1)

2.1.11.3 Tris-HCl SDS stock buffers

The Tris-HCl SDS stock buffers used for the preparation of the resolving and stacking gels were purchased from National Diagnostics Ltd. ProtoGel™ Resolving Buffer solution consisted of 1.5 M Tris-HCl (pH 8.8) and 0.1 % (w/v) SDS, whereas ProtoGel™ Stacking Buffer consisted of 0.5M Tris-HCl (pH 6.8) and 0.1 % (w/v) SDS.

2.1.11.4 Resolving gel, pH 8.8

- 10 % (w/v) bis-acrylamide
- 25 % (v/v) resolving buffer
- % (w/v) APS
- % (v/v) TEMED

2.1.11.5 Stacking gel, pH 6.8

- 4 % (w/v) bis-acrylamide
- 25 % (v/v) stacking buffer
- 0.075 % (w/v) APS
- 0.15 % (v/v) TEMED

2.1.11.6 Running buffer (10×), pH 8.3

- 0.25 M Tris-HCl
- 1.92 M glycine
- 1 % (w/v) SDS

2.1.12 Protein molecular weight markers

Precision Plus Protein Standards (All Blue) were purchased from Bio-Rad Laboratories Ltd. and contained ten proteins of 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 250 kDa. The 25 kDa, 50 kDa and 75 kDa proteins serve as reference bands because they are three times as intense as the other bands.

2.1.13 Western blotting and immunodetection solutions

2.1.13.1 Electroblotting transfer buffer

- 25 mM Tris-HCl (pH 8.3)
- 192 mM glycine
- 20 % (v/v) methanol

2.1.13.2 Blocking solution

5 % (w/v) skimmed milk powder in PBS-Tween-20 [0.1 % (w/v)]

2.1.13.3 Primary antibody incubation solution

5 % (w/v) skimmed milk powder in PBS-Tween-20 [0.1 % (w/v)]

2.1.13.4 Secondary antibody incubation solution

PBS-Tween-20 [0.1 % (w/v)]

2.1.14 Immunofluorescence solutions

2.1.14.1 Fixative

4 % paraformaldehyde (PFA) in PBS

2.1.14.2 Blocking and permeabilising solution

Dissolved in PBS:

0.1 % (v/v) Triton X-100

5 % PBS

2.1.14.3 Antibody diluent

Dissolved in PBS:

10 % blocking solution

2.1.15 Antibodies

Primary antibodies and secondary antibodies used are listed in tables 2.2 and 2.3 respectively.

Table 2.2. Primary antibodies used for immunofluorescence and western blotting (WB)

Antibody	Species	Epitope	Origin	Dilution WB	Dilution immunofluorescence
3B1	Mouse monoclonal	RRM3 of CELF proteins	Santa Cruz Biotechnology	1/1000	1:300
mAb104	Mouse monoclonal	RS domain of SR proteins	ATCC®	1/10	-
GAPDH	Mouse monoclonal		Sigma-Aldrich	1/1000	-
β-actin	Mouse monoclonal		Sigma-Aldrich	1/500	-

Table 2.3. Secondary antibodies used for immunofluorescence and WB

Name	Antibody	Origin	Dilution WB	Dilution immunofluorescence
IRDye ™800 conjugated	Rabbit polyclonal	Rockland	1/10000	-
Alexa Fluor® 680 conjugated	Goat anti-mouse Monoclonal	Molecular Probes	1/10000	1/5000

2.1.16 Cell culture

All medium used for the culture of cell lines was supplemented with:

2 mM L-glutamine

Penicillin (100 units/ml)

Streptomycin (100 µg/ml)

2.1.16.1 Medium for Chinese hamster ovary (CHO) cells

Nutrient mixture Ham's F-12 (HAM)

10 % (v/v) FBS

2.1.16.2 Medium for SH-SY5Y cells

DMEM/nut mix F-12

15 % (v/v) FBS

2.1.16.3 Medium for HeLa cells

DMEM 4.5 g/l glucose

10 % FBS

2.1.16.4 Hank's balanced salt solution (HBSS)

HBSS was purchased from Invitrogen Ltd. as a 1× solution without calcium chloride, magnesium chloride or magnesium sulphate. This solution was used to maintain cells for short periods of time during passage and washing before tryptinisation.

2.1.16.5 Trypsin solution

Trypsin-EDTA (TE) was purchased from Invitrogen Ltd. as a solution containing 0.05 % (w/v) TE in PBS.

2.1.16.6 OPTIMEM™ solution

Optimem was purchased from Invitrogen as a 1× solution containing:

HEPES buffer

2.4 g/l sodium bicarbonate

Hypoxanthine

Thymidine

Sodium pyruvate
L-glutamine
Growth factors
Phenol red reduced to 1.1 mg/l

2.1.16.7 Tranfection reagents

Transfection was performed with Lipofectamine and Plus reagent (Invitrogen).

2.1.17 Miscellaneous stock solutions

2.1.17.1 PBS

PBS tablets were purchased from Sigma-Aldrich Co. Ltd. One tablet was dissolved in 200 ml of ddH_2O to give a final concentration of:
0.01 M phosphate buffer pH 7.4
0.0027 M potassium chloride
0.137 M sodium chloride

2.1.18 Human brain tissue

Frozen human brain tissue was obtained post-mortem from neurologically normal (controls) and individuals with moderate/severe AD (Braak stages V- VI) from the London Neurodegenerative Diseases MRC Brain Bank, Institute of Psychiatry, King's College London (Ethical Committee Protocol #: 174/02). Brain tissue was dissected from subjects with AD; with and without TDP-43 inclusions. Staging of AD cases and definitive diagnosis for AD cases with and without TDP-43 inclusions were made according to established clinical criteria and neuropathological criteria by pathologists and neuropathologists at the MRC Brain Bank. Dissection was carried out by Dr Tibor Hortobágyi and Dr Claire Troakes. Five brain regions were dissected where possible.

Five brain areas from each case were selected for study. Brain regions selected include Frontal lobe BA 8/9, Temporal lobe BA 21, Amygdala, Hippocampus and Cerebellum. These brain regions develop neurofibrillary tangles (NFTs) during

the early stages of disease are heavily affected with tangles and plaques (Braak and Braak, 1995). The cerebellum is used as a control region from affected individuals because it is largely unaffected in AD with fewer occurrences of NFTs and functional damage in AD (Braak and Braak, 1995). Tissue was collected and stored in tubes containing 200 mg and 100 mg brain tissue from 31 human brains and stored at -70 °C until use. 100 mg of tissue was put directly into matrix lysing-D tubes (Qbiogene) for RNA extraction using the FastPrep sample preparation system (RNA extraction from human post-mortem brain tissue is listed in Table 2.4).

A significant factor that affects RNA quality from post-mortem human brain tissue is the pH of the tissue (Kingsbury et al., 1995). The pH is accepted as an indicator for the duration and severity of the agonal state and the simulataneous hypoxia leading to acidiosis. An exteneded period of agonal state (which causes hypoxia) may lead to reduced RNA and protein integrity and content (Chevyreva et al., 2007). All our brain samples had a pH within the acceptable range (pH 6.1 – 7.0) reported for the obtainement of good quality RNA (Bahn et al., 2001) (Figure 2.4). Tissues with low or acidic pH (pH < 6.0) produce fragmented RNA reported to have a strong correlation with tissue pH and RNA quality. This is true for both control and pathological brain tissue (Bahn et al., 2001).

Eight controls (age range 66 – 81, mean ± STDEV. 73.5 ± 13.29), fifteen ADTDP43- cases (age range 69 – 97, ± 8.50) and eight ADTDP43+ cases (age range 73-97, ± 7.88) (Table 2.4). Control samples analysed were void of any pathology seen in AD brain such as tau aggregates or amyloid plaques or symptoms and AD cases are all sporadic cases with advanced disease.

Table 2.4. Brain samples dissected and collected from IOP Brain Bank.

BB Case No.	PM diagnosis	Age	sex	PMD (hours)	pH
A249/07	ADTDP43+	74	M	69	6.77
A190/07	ADTDP43+	88	F	73	6.98
A221/07	ADTDP43+	86	F	47	6.67
A205/07	ADTDP43+	73	M	24.5	6.37
A349/08	ADTDP43+	86	F	14	6.56
A348/07	ADTDP43+	85	M	16	6.4
A063/09	ADTDP43+	89	M	62	6.82
A076/09	ADTDP43+	97	M	16.5	6.18
A037/04	ADTDP43-	96	F	39	6.62
A039/02	ADTDP43-	90	F	4	6.63
A331/07	ADTDP43-	80	F	12.5	6.48
A098/04	ADTDP43-	84	M	73	6.86
A191/07	ADTDP43-	69	F	16.3	7.43
A192/07	ADTDP43-	96	F	19	6.57
A210/05	ADTDP43-	84	F	<24	6.57
A240/06	ADTDP43-	97	F	12	6.9
A141/07	ADTDP43-	80	M	41	6.33
A050/04	ADTDP43-	91	F	28.5	6.64
A058/07	ADTDP43-	81	M	74	6.76
A160/06	ADTDP43-	71	M	32	6.42
A187/07	ADTDP43-	82	F	69	6.67
A122/04	ADTDP43-	86	M	24	6.72
A065/04	ADTDP43-	91	F	28.5	6.19
A140/07	Control	81	F	16.5	6.1
A239/03	Control	78	M	9.5	6.63
A359/08	Control	80	F	3	6.4
A308/09	Control	66	M	52	6.66
A292/09	Control	43	F	43	6.66
A123/09	Control	78	M	24	6.28
A113/09	Control	18	M	24.5	6.44
A048/09	Control	81	M	18	6.72

BB case no. Brain Bank case number; PM diagnosis Post mortem diagnosis; Age = age at death; (data from Medical Research Council Genetic Resource for Late-onset AD); PMD Post mortem delay; ADTDP43+ Alzheimer's disease with TDP-43 inclusions;ADTDP43-,Alzheimer's disease without TDP-43 inclusions.

2.2 Methodology

2.2.1 RNA extraction

2.2.1.1 RNA extraction from postmortem human brain

All RNA methods were carried out with RNase free plasticware and RNase free non-DEPC treated water (Ambion).

Total RNA was isolated from frozen blocks of human brain. Samples were homogenised in matrix lysing-D tubes in conjunction with the Qbiogene FastPrep sample preparation system. Total RNA was extracted from the homogenised samples using the Qiagen RNeasy lipid tissue kit according to the manufacturer's protocol. RNase-free tubes (for storage of RNA at -70 ^{o}C), lysis solution, buffers and RNase free water (for elution) are provided with the Qiagen RNeasy lipid tissue kit.

Briefly, 1 ml of 4 °C Qiazol lysis solution (containing phenol and guanidine thiocyanate) was added to approximately 100 mg of brain tissue inside lysing matrix-D tubes (Qbiogene). Matrix D tubes contain ceramic beads that homogenize the tissue when used in conjunction with the FastPrep 24 machine. This machine is a high-speed bench top homogenising device that disrupts tissues. RNA was eluted in 40 µl of RNase-free water. Isolated RNA was stored at -70 ^{o}C until use.

2.2.1.2 RNA isolation from rat brain tissue

Total RNA was isolated from frozen blocks of rat brain (snap frozen in liquid nitrogen) using the Qiagen RNeasy lipid tissue kit using the same protocol as for the human brain samples. However, we used a mortar and pestle instead of the matrix lysing-D tubes for homogenisation of these samples. Approximately 100 mg of brain tissue (kept on dry ice before use) was homogenised in 1 ml of 4 °C

Qiazol lysis solution. The brain tissue was homogenised with approximately 20 strokes, on ice and RNA extracted as for the human samples.

2.2.1.3 Total RNA isolation from HeLa and SHSY5Y cells

HeLa and SHSY5Y cells were grown in a monolayer in 6-well plates and lysed with 1 ml of Trizol® reagent (Invitrogen Ltd.) added directly onto each well. Cell lysis was achieved by passing the cells through a pipette several times. Homogenised cells were incubated at room temperature for 5 min to break nucleic acid-protein complexes in the cells. 200 µl chloroform was added to the homogenate and vortexed for 15 s. Thereafter, centrifugation of the homogenate was carried out at 12, 500 rpm for 15 min at 4 ^{o}C. The clear layer at the top phase containing RNA was transferred into a new tube while avoiding the protein-containing interphase. RNA was precipitated with 500 µl isopropanol by incubating for 15 min at room temperature. The resultant RNA pellet obtained after centrifugation at 12, 500 rpm for 15 min at room temperature was washed three times with 1 ml 75 % (v/v) ethanol before resuspension in 50 µl of RNase-free water. The isolated RNA was stored at -70 ^{o}C.

2.2.1.4 Quantification and DNase treatment of RNA

DNase treatment of isolated RNA removed genomic contaminant DNA from the RNA sample. DNA-free™ kit (Applied Biosystems) was used to treat RNA according to the manufacturer's instructions. Briefly, 5 µl of 10× DNase buffer and 1 µl rDNase were added to the RNA and mixed gently. The mixture was then incubated at 37 ^{o}C for 30 min. To stop the reaction, 5 µl of DNase inactivation reagent was added while gently mixing and incubating for 2 min at room temperature. The DNase and inactivation reagents were removed by centrifugation (10, 000 g for 1.5 min) and the resultant RNA was transferred to a new tube. RNA concentration was determined with Nanodrop spectrophotometer (Thermo Scientific) using the manufacturer's software. The pedestal was first cleaned using lint-free cloth. The machine was self-caliberated after each use and zeroed using appropriate buffer in which the nucleic acid is stored. 1.5µl of each undiluted total RNA samples was analysed, recording the concentration in ng/µl.

Also, the OD260/OD280 readings were between 1.8 and 2.1 meaning that there is little or no protein contamination in a RNA sample.

2.2.1.5 RNA integrity

RNA integrity number (RIN) was measured with the Agilent RNA 6000 Pico kit using the Agilent 2100 Bioanalyser. Chips were prepared for all human brain RNA according to the manufacturer's instructions. The RNA samples and ladder were prepared and kept on ice until use. RIN was measured recorded in Table 2.5. RIN number less than ~ 5 was excluded from data analysis because it is below the acceptable range for good RNA quality.

Briefly, 550 µl of the RNA 6000 Pico gel matrix was placed on a spin filter, and centrifuged at 1, 500 g for 10 min at room temperature. This was then divided into 65 µl aliquots. After the addition of 1 µl of the RNA 6000 Pico dye concentrate to the 65 ul aliquot of filtered gel, the gel-dye mix was vortexed and centrifuged at 13, 000 g for 10 min. A RNA ladder consisting of a mixture of RNA with known concentration was run after the addition of 5 µl marker to the ladder and sample wells. 1 µl (3 ng/µl) RNA of each RNA sample was added in the 11 designated sample wells, followed by the addition of 1 µl of diluted RNA 6000 ladder in the ladder well. The kit consists of interconnected microchannels that separate RNA fragments (18S and 28S for eukaryotic RNA) based on their size when driven electrophoretically. The eukaryotic 18S and 28S RNA are detected and plotted on an electropherogram graph.

To determine the RIN number, the area under the RNA electropherogram is calculated and converted to concentration values using the ladder's concentration/area ratio.

Table 2.5. RNA integrity (RIN) from brain regions of human brain.

	BBCase	Code	RIN FC	RIN TC	RIN A	RIN H	RIN C
ADTDP43+	A249/07	T2	6.8	7.4	4.3	4.8	7.8
	A190/07	T3	2.3	2.4	2.2	2.6	2.2
	A221/07	T4	2.3	2.3	2.8	2.4	4.9
	A205/07	T5	4.5	3.8	5.9	4.8	6.4
	A349/08	T6	5	5.7	3.1	3.6	6.4
	A348/07	T7	7	7.6	6.5	6.3	5.2
	A063/09	T8	5.3	5.5	5	3.2	6
	A076/09	T9	5.1	4.6	4.2	4.2	6
ADTDP43-	A037/04	A1	6.7	7.7	5.5	3.1	5
	A039/02	A2	4.7	4.6	4.3	3.6	6.4
	A331/07	A3	6.8	4.3	6.1	4.7	6.9
	A098/04	A4	5.3	4.2	4.7	3.8	6.7
	A191/07	A5	7.6	6.9	7.4	6.6	8.5
	A192/07	A6	5.2	4.5	5.6	5.2	5.8
	A210/05	A7	4.2	2.5	4.6	2.7	5.3
	A240/06	A8	6.1	5	4.3	4.4	2.7
	A141/07	A9	5.9	4.3	5.4	5.5	6.9
	A050/04	A10	5.2	5.4	5.9	1.1	6.1
	A058/07	A11	4.9	4.7	2.6	3	7
	A160/06	A12	3.7	4.3		5.8	7.3
	A187/07	A13	5	3.1	3.4	4.3	5.4
	A122/04	A14	6	5.6		4.1	7.8
	A065/04	A15	5.1	5.3	5.7	6.1	6.1
Control	A140/07	C1	2.4	2.3	2.1	2.4	2.3
	A239/03	C2	4.2	5.2	4.7	6	6.8
	A359/08	C3	7.8	5.8	7.4	7.1	7.2
	A308/09	C4	4.3	4.5	2.6	3.8	4.8
	A292/09	C5	6.7	7.8	7	6.9	8.4
	A123/09	C6	4.9	5.8	4.8	5.6	3.2
	A113/09	C7	2.5	2.6	3.6	3.4	3
	A048/09	C8	4.5	6.1	4.8	4.8	7.2

RIN FC= Frontal cortex; RIN TC= Temporal cortex; RIN A= Amygdala; RIN H= Hippocampus; RIN C= Cerebellum. RIN is measured in arbitrary units from 0-10 with 10 being RNA of high integrity. Missing values indicate where no tissue was available.

2.2.1.6 Reverse transcription of RNA

Reverse transcription (RT) was performed using reagents from Taqman RT Kit (GE Heathcare). 0.5 μg of total RNA was reverse transcribed in a total volume of 10 μl. Master mixes for each reaction are as follows:

Reagent	Volume (μl)	Final Concentration
10× Buffer	1	1/10 dilution
$MgCl_2$ (25 mM)	2.2	5.5 mM
dNTP mix	2	500 μM of each dNTP
Oligo-dT	0.5	2.5 μM
RNase inhibitor	0.2	0.4 U/μl
Reverse transcriptase (50 U/μl)	0.25	1.25 U/μl

A G-storm GS1 thermal cycler was used to perform the RT reaction with conditions as follows:
25 °C for 10 minutes – oligo-dT hybridization
48 °C for 30 minutes – reverse transcription
95 °C for 5 minutes – denature reverse transcriptase
4 °C for storage

2.2.1.7 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with GoTaq polymerase reagents (Promega) using primers listed in Table 2.1. Master mixes for multiple reactions were prepared to avoid variability. For one reaction, the PCR mix was as follows:

Reagent	Volume (μl)	Final Concentration
5× Buffer	10	1/5 dilution
dNTP mix (2.5 mM)	4	200 μM
Forward primer	5	200 μM
Reverse primer	5	200 μM
H_2O	15.5	n/a
Taq polymerase	0.5	0.05 U/μl

40 μl of the PCR mix was mixed with 10 μl of the RT reaction. PCR controls without RNA (no template condition) and/or without RT (no RT condition) as well as water controls were routinely carried out to detect DNA contamination in the RT-PCR reaction. The PCR products are analysed at the end of the reaction hence the name 'end-point PCR' in contrast to real-time PCR. Gel electrophoresis was used to access the presence of PCR products according to their size. The PCR reaction was performed with the G-Storm GS1 thermal cycler according to parameters used of each primer set. For each specific transcript, the parameters are thus:

GAPDH primers

1 cycle	Denaturation	95 °C for 5 min
	Denaturation	94 °C for 30 s
28 cycles	Annealing	62 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

β-actin primers

1 cycle	Denaturation	95 °C for 5 min
	Denaturation	95 °C for 30 s
28 cycles	Annealing	55 °C for 30 s
	Extension	72 °C for 45 s
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

SC35 primers

1 cycle	Denaturation	95 °C for 2 min
	Denaturation	94 °C for 30 s
40 cycles	Annealing	65 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

SRp40 primers

1 cycle	Denaturation	95 °C for 5 min
	Denaturation	94 °C for 30 s
28 cycles	Annealing	62 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

SRp55 primers

1 cycle	Denaturation	95 °C for 5 min
	Denaturation	94 °C for 30 s
28 cycles	Annealing	62 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

Endogenous tau exon 10 primers

1 cycle	Denaturation	95 °C for 2 min
	Denaturation	94 °C for 30 s
35 cycles	Annealing	65 °C for 30 s
	Extension	72 °C for 20 s
1 cycle	Extension	72 °C for 10 min
1 cycle	Storage	4 °C for storage

CELF1 primers

1 cycle	Denaturation	95 °C for 6 min
	Denaturation	95 °C for 30 s
35 cycles	Annealing	57.4 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Storage	4 °C for storage

CELF3 primers

1 cycle	Denaturation	95 °C for 6 min
	Denaturation	94 °C for 30 s
35 cycles	Annealing	57.1 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Storage	4 °C for storage

CELF4 primers

1 cycle	Denaturation	95 °C for 5 min
	Denaturation	95 °C for 30 s
35 cycles	Annealing	57.4 °C for 30 s
	Extension	72 °C for 1 min
1 cycle	Storage	4 °C for storage

2.2.1.8 Quantitative RT-PCR

Taqman RT Kit (GE Healthcare) was used to synthesize the first strand cDNA as described above. In contrast to 'end-point' PCR, the principle of quantitative reverse transcription polymerase chain reaction (RT-qPCR) also known as real-time RT-PCR allows the amplification and fluorescent detection step to be performed in one machine, in a single tube while recording the data. This is usually done after each PCR cycle. The Chromo 4^{TM} (Bio-Rad) which supports the Opticon Monitor^{TM} version 3.1 system was used to measure accumulation of PCR products during amplification with fluorescent dyes. The fluorophore used in this study was SYBR Green I (Roche Diagnostics).

Briefly, the sequence-independent detection assay we used works with the principle that SYBR Green I bind to all double-stranded DNA (dsDNA) molecules irrespective of the sequence. The Chromo 4^{TM} machine detects SYBR Green I fluorescence. SYBR Green I does not fluoresce when free in solution but after binding to dsDNA, its fluorescence emission is enhanced as a result of conformational changes in the dye. Hence, the increase in SYBR Green I signal (measured at 530 nm) was correlated with the amount of PCR products amplified during the reaction.

RT-qPCR was performed on 20 ng template cDNA with The Chromo 4^{TM} machine using the FastStart SYBR Green Master (Rox) (Roche Diagnostics) according to the manufacturer's manual. The RT reaction was performed as described above (Section 2.2.1.6). For each cDNA template, primers for GAPDH and β-actin were loaded in different wells on the same plate. For example, SC35 had one 10 μl reaction and a separate one for GAPDH and β-actin on the same 96 well plate. Master mixes for multiple reactions were prepared to avoid variability. For one reaction, the RT-qPCR mix was as follows:

Reagent	Volume (μl)	Final Concentration
Fast start SYBR green (Roche)	5	1×
Forward primer	0.5	10 μM
Reverse primer	0.5	10 μM
H_2O	2	N/A
cDNA	2	10 ng/μl

8 μl of the PCR mix was mixed with 2 μl of the cDNA solution. Two PCR controls without cDNA (with water) are performed for each transcript as well as each house-keeping gene to detect contamination in the RT-qPCR reaction. For each specific transcript, the PCR conditions were 3 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and melting curves from 60 to 90 °C every 0.5 °C. Amplification of the single gene transcript was confirmed by monitoring the

dissociation curve. Suitable baseline range and threshold levels were set after visual inspection of the amplification curves. All measurements were performed in duplicates. GAPDH and β-actin RNA levels were used for normalisation. The relative quantification method was employed for quantification of target molecules, in which the ratio between the amount of target molecule and the house-keeping gene molecule within the same sample was calculated.

2.2.1.9 Data Analysis

Quantification of RT-PCR and RT-qPCR products

Data were expressed as percentage mean ± standard error of mean (SEM) unless otherwise specified of 4R tau amplicon in total tau (4R + 3R tau). Differences between two groups for 4R tau expression were determined by unpaired Student's *t* test. For RT-qPCR products, data were expressed as mean ± S.E.M. Differences between controls, ADTDP43- and ADTDP43+ groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *P*-value less than 0.05 denoted statistically significant difference.

2.2.1.10 PCR primers validation

The primers used for RT-qPCR for all genes of interest in human brain were designed on the basis of the GenBank cDNA sequence using the primer designing program Primer3. The primers used are listed in Table 2.1. Each primer was tested for specificity using the NCBI Blast program and all were shown to be gene-specific and free of non-specific homology. The primers were then tested using 'end-point' PCR amplification protocol to amplify a product from a human cDNA template. In all cases, a single product of the expected size was amplified (see results section Chapters four and five). In addition, 100 % match was detected after sequencing for all transcripts according to Ensemble database. Finally, the primers were tested under the RT-qPCR protocol and in all cases; a single melting temperature curve was produced which indicates that only a single amplification product is present (Figure 2.1 and appendix 1.2).

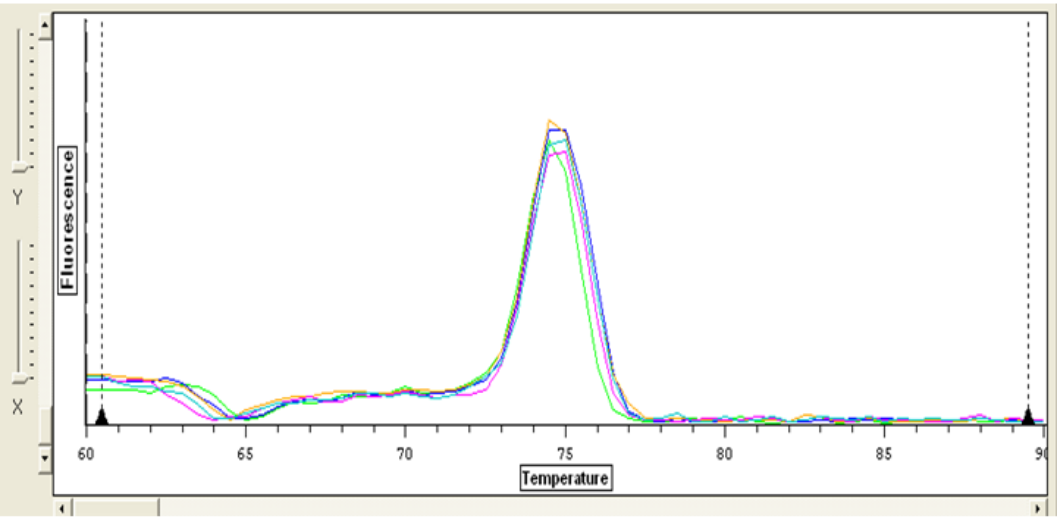


Figure 2.1. A typical RT-qPCR melting curve

2.2.2 DNA agarose gel electrophoresis

1 and 2 % (w/v) agarose gels were prepared using electrophoresis grade agarose (Sigma). Agarose was melted in 100 ml 1× TAE buffer using a microwave oven. The DNA intercalator, ethidium bromide, (Sigma, 5 µl of 10 mg/ml) was added to the agarose gel prior to cooling. Melted gel was allowed to set in a horizontal casting tray with a suitable well comb. Set gels were placed in a Hybaid horizontal electrophoresis tank (Thermo Scientific) containing 1× TAE buffer. PCR samples were loaded into the wells and run with appropriate DNA markers. The electrophoresis reaction was performed at 125 V until the dye front had reached the bottom of the gel.

2.2.2.1 DNA extraction from agarose gels

DNA bands were excised from the gel using a clean scalpel blade and purified using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The principle of action is based on a DNA absorption membrane with DNA eluted from high salt conditions using a low salt buffer.

2.2.2.2 Acquisition of agarose gel images

Images were acquired from the UVP BioDoc-It^{TM} transilluminator with a digital camera. Gels were viewed at a wavelength of 302 nm.

2.2.2.3 Acquisition of agarose gel images and analysis with the Odyssey imager

Images were acquired by scanning whole DNA gels with the Odyssey scanner to upload images of DNA fragments containing the infrared-dye (IRD 700) (MWG Biotech) labelled primers (Figure 2.2) visible by scanning with the 700 nm channel. To quantify alternatively spliced tau isoforms accurately, our laboratory developed a sensitive method where alternatively spliced variants were visualised and measured using the Odyssey infrared imaging system. This method was employed because visualisation of DNA fragments using ethidium bromide can prove to be problematic for quantifying small differences between alternatively spliced isoforms. Ethidium bromide is a DNA intercalator that only measures the mass of DNA rather than the number of molecules. This therefore makes it difficult to compare relative molecular abundances of DNA fragments with different sizes. For instance, a DNA fragment containing an alternative exon will have more ethidium bromide staining compared to one without the alternative exon. This makes measurements of isoform ratio incorrect for investigating changes in alternatively spliced isoforms. In contrast, using the labelled primers accurately measures changes in isoform ratio compared to DNA intercalators. With this method, the signal intensity detected from the primer label is independent of the size of the DNA.

Existing tau 9 – 13 primers were modified by attaching a fluorophore at the 5' end of the forward primer (Figure 2.2) (Table 2.1). The IR-700 labelled primers were incorporated into DNA fragments using existing PCR protocols that were optimised to account for the proportion of labelled to unlabelled forward primer. The intensity of the DNA bands was quantified using the LiCor manufacturer's software. Analysis was performed in triplicate and significant differences were analysed by unpaired Student's *t* test.

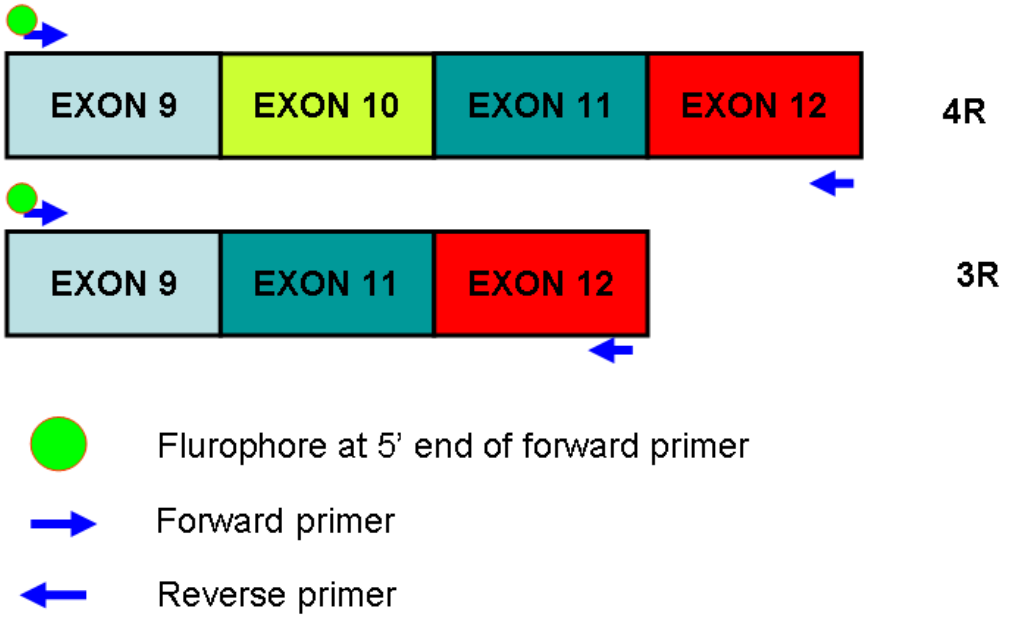


Figure 2.2. Position of tau 9 – 13 primer in the *MAPT* gene.

Schematic structure of *MAPT* gene comprising of tau exons 9-10-11-12-13 and full-length introns 9, 10, 11 and 12. The structure of the gene products after alternative splicing are schematically indicated (4R and 3R). Forward and reverse primers are indicated with blue arrows. The Forward primer in exon 9 has a fluorophore attached to its 5' end (green circle).

2.2.3 Cloning

Ligation

Ligation of cDNA insert (extracted PCR product) and vector was performed overnight on slush ice with 1 μl T4 ligase and 1 μl ligase buffer (Promega) in a total volume of 10 μl. 100 ng of vector DNA was used and the amount of insert DNA calculated as follows:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{Kb size of vector}} \times \frac{\text{molar ratio of insert}}{\text{vector}}$$

The six PCR products resulting from primer sets SC35, SRp40, SRp55, CELF1, CELF3 and CELF4 were validated (Tau 9-13, Total Tau, GAPDH and β-actin primers have previously been validated). PCR products were cut out of 2 % agarose gels; DNA was extracted from agarose and purified using the QIAquick gel extraction kit (Qiagen) (section 2.2.2.1). The DNA was ligated into pGEM-T easy vectors (Promega).

2.2.4 Transformation of *E.coli* cells

20 ng of plasmid DNA was transformed into 50 μl of competent JM109 *Escherichia coli* (*E. coli)* cells (Promega) and incubated on ice for 20 min. The *E.coli* cells were subsequently heat-shocked at exactly 42 °C for 45 s and returned to ice for 2 min. 950 μl of sterile LB medium without antibiotic was added to the bacterial suspension and incubated at 37 °C for 1 hour to allow for antibiotic resistance gene expression. Bacteria were centrifuged for 5 min at 8000 rpm and re-suspended in 50 μl LB broth. Cells were streaked out onto LB agar plates (LB/ampicillin/IPTG/X-Gal) and placed in an incubator overnight at 37 °C to isolate individual colonies.

IPTG/X-Gal allows colonies containing the DNA insert to be selected by blue/white selection. The pGEM-T easy vector contains a LacZ gene that codes

for the production of an enzyme beta-galactosidase. Within the LacZ gene there are multiple cloning sites where the plasmid is cut and the DNA insert is added. This produces a plasmid with foreign DNA located within the LacZ gene. When the LacZ gene with the foreign DNA insert is translated into an enzyme, the translated protein product is disrupted and a non-functioning enzyme is produced, hence a white colony. White colonies containing the DNA insert were selected and grown in 5 ml of LB broth/ampicillin in a Unitron Infors AG shaker, shaking 225 rpm, 37 °C overnight.

2.2.5 Plasmid DNA digestion by restriction endonucleases

The presence of the cDNA insert was confirmed with small-scale restriction enzyme analytical digest and agarose gel electrophoresis before sequencing. 1 μl of 1 unit/μl restriction enzyme, EcoR1 (Promega) was used to digest cDNA inserts from 1 μg of plasmid DNA with 2 μl 10× buffer with ultra-pure water to produce a final volume of 20 μl. Reactions were performed at 37 °C for 2 h to allow for complete digestion of plasmid DNA followed by a denaturation step at 65 °C for 10 min. Digested DNA was then run in a 2 % agarose gel electrophoresis to confirm the presence of the cDNA insert in the purified plasmid DNA (Section 2.2.2).

Correct orientation of cDNA insert was confirmed by sequencing (MWG) in addition, to further confirm successful cloning. The insert DNA sequence was compared and validated with cDNA sequences downloaded from Ensemble database.

2.2.6 Cell culture

All materials used for cell culture were purchased sterile or sterilised by autoclaving at 121 °C for 15 minutes. Monthly tested of cells were performed to detect mycoplasma contamination. All procedures were performed in class II microbiological safety cabinets.

2.2.6.1 Cell lines

Throughout this study, cell lines of human origin were used. The following cell lines were used in this study:

- *SH-SY5Y* - A third generation human neuroblastoma cell line originated from the SK-N-SH line derived from a bone marrow biopsy in 1970 (Biedler et al., 1973; Ross et al., 1983)

- *HEK-293* - Human cell line of embryonic kidney origin originated by the transformation of normal human embryonic kidney cells with sheared adenovirus 5 DNA.

- *HeLa* – A human cancer cell line of cervical cancer origin.

- The mA104 hybridoma cells were used to produce mAb104 antibody.

2.2.6.2 Culture of cell lines

Cell lines were cultured in medium supplemented with FBS, glutamine and penicillin/streptomycin. All cell lines were maintained in humidified atmosphere containing 5 % CO_2/ 95 % air were used to maintain cells in a Heraeus Hera-cell incubator at 37 °C. SH-SY5Y and HEK293 cells were passaged at a sub-confluent stage and disposed off after passage 35. HeLa cells were maintained indefinitely.

The mA104 hybridoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % (v/v) FBS. After collection of the antibody, the cell pellets were resuspended in the appropriate volume of medium for further antibody production at approximately 10^6 cells/ml. The growth medium was replaced every 3 to 4 days to maintain the cells.

2.2.6.3 Passaging of cell lines

Cells were maintained by passaging when approximately 80 % confluent. Culture medium was aspirated and the cells washed with HBSS. Cells were then trypsinised by the addition of Trypsin-EDTA solution and incubating at 37 °C until cells were fully detached. Cells were then re-suspended in culture medium to inactivate the trypsin. Cells were then recovered by centrifugation at room temperature at 1000 rpm for 5 minutes using a Sorvall® Legend T centrifuge, re-suspended in 10 ml of fresh medium and reseeded at 1/10 dilution.

2.2.6.4 Production of mAb104

Mouse hybridoma cells which produce the monoclonal antibody 104 (mAb104) were purchased from ATCC (ATCC ®). The antibody was harvested, when the growth medium was yellow, the cell suspension was collected in 50 ml centrifuge containers by spinning down at 1, 000 g for 20 min at room temperature. The supernatant was then transferred to fresh tubes prior to addition of 0.01 % sodium azide to prevent bacterial growth. mAb104 was then frozen at -20 ^{o}C until use.

2.2.6.5 Transfection of cell lines

Lipofectamine and Plus Reagent transfection

HeLa cells were transfected with Lipofectamine Plus™ reagent (Invitrogen Ltd.) in 6-well plates. Two solutions were made in sterile microcentrifuge tubes:

Solution A: For each transfection, 2 µg DNA (plasmid), 150 µl OPTIMEM™ and 12 µl Lipofectamine plus reagent were mixed together and incubated for 20 min at room temperature.
Solution B: For each transfection, 6 µl Lipofectamine was diluted in 1.2 ml serum-free Optimem.

The two solutions (A and B) were mixed gently, and incubated at room temperature for 20 min. The cells were washed once with 2 ml serum-free medium.

For each transfection, 1 ml serum-free medium was added to each tube containing the lipid-DNA complexes. The solution was gently mixed and the diluted complex solution was spread over the surface of the washed cells. The cells were incubated for 3 h at 37 °C/ 5 % CO_2. After 3 hours the transfection mixture was removed and replaced with the normal volume of fresh medium and incubated for 24 h. Cells were harvested 24 h after transfection to extract RNA.

2.2.7 Immunofluorescence

HeLa cells were washed with 1× PBS at 37 °C and fixed using 4 % PFA in PBS for 10 min at room temperature. Cells were then washed three times with 1× PBS followed by permeabilising with 1× PBS containing 0.1 % Triton X-100 for 30 min at room temperature. Coverslips were removed from the plastic wells and inverted onto primary antibody (3B1 and anti-SR protein antibody, 104) for 1 h at room temperature. Coverslips were then washed three times with 1× PBS before being incubated with a species-specific secondary antibody at room temperature in the dark. Coverslips were washed again three times with 1× PBS with the final wash containing Hoechst 33258 (180 mM). Finally coverslips were mounted onto glass slides using Dako-Cytomation fluorescent mounting medium. Slides were viewed with a Zeiss axioskop fluorescence microscope with appropriate filters and imaged using the Metamorph software package (Molecular Devices, Sunnyvale CA).

2.2.8 Protein extraction from cell lines

Cell lysates were prepared from 6-well plates when 85 % confluent. The cells were kept on ice and washed three times with ice-cold PBS. Cells were then scrapped into 1× Laemmli sample buffer at 100 °C. The cell lysates were

then boiled for 5 min at 100 °C and centrifuged for 5 min at 1,300 g at room temperature before analysis by SDS-PAGE.

2.2.9 Preparation of rat brain homogenate

A female rat was sacrificed by overdose of CO_2 followed by severing of the spinal cord. The brain was then removed and was quickly snap frozen in liquid nitrogen. Whole brain was homogenised in 1 × sample buffer containing protease inhibitors using a pre-chilled glass homogeniser with approximately 20 strokes. Brain homogenate was aliquoted into 1.5 ml tubes and kept on ice for 5 min.

Brain homogenate was then transferred to pre-chilled centrifuge tubes and centrifuged 37,000 g for 1 h at 4 °C using a Beckman-Coulter Optima TLX Ultracentrifuge. The pellet was discarded and the clear supernatant was used as the starting material. Samples were then boiled for 5 min and centrifuged before analysis by SDS-PAGE.

2.2.10 Preparation of Human brain homogenate

Brain tissue was obtained from the London Neurodegenerative Diseases Brain Bank, Institute of Psychiatry, King's College London (temporal cortex of normal control groups: no tau deposits n=3). Ethical approval was obtained from the local Ethics Committee.

Human brain samples were kept on dry ice before use. 100 mg of frozen human brain tissue was homogenised using a microcentrifuge tube and microcentrifuge pestle (20 strokes) in 5ml of boiling (100 ^{o}C) 1× Laemmli sample buffer. The homogenate was centrifuged for 1 h at 47, 000 g at 4 ^{o}C using a Beckman-Coulter Optima TLX Ultracentrifuge. The clear layer in the middle (supernatant) was then transferred to a new tube.

Due to the presence of high concentrations of SDS and β-mercaptoethanol, protein assays (BCA and Bradford) could not be carried out. A GAPDH (a classical

standard for loading control) antibody was as a protein loading measure. The brain homogenates were prepared for western blotting as follows. 0.0125 % (w/v) bromophenol blue was dissolved in ddH_2O and added to the homogenised brain samples to make the final concentration of sample buffer, 1×. For SDS-PAGE, the brain homogenates were heated at 100°C for 5 min prior to centrifugation for 5 min at 1, 300 g at room temperature prior to loading.

2.2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on one-dimensional gels using the Mini-Protean Tetra-cell gel system (Bio-Rad Laboratories Ltd.) based on the method of Laemmli (1970). 10 % (w/v) bis-acrylamide resolving gel was prepared and following polymerisation a 4 % (w/v) bis-acrylamide stacking gel was poured on top of the resolving gel. An appropriate well comb was inserted and the gel allowed to polymerise. Unless otherwise stated 10 μl of samples were loaded onto the gels and run in 1× SDS-PAGE running buffer at 150 V until the bromophenol blue dye front had reached the bottom of the gel. An appropriate molecular weight protein ladder was run in parallel.

2.2.12 Western blotting (WB)

Protein separated on SDS-PAGE gels were transferred to a 0.45 μm pore size nitrocellulose membrane (Whatman® Protran®) using a Trans-Blot Semi-Dry Transfer Cell™ (Bio-Rad Laboratories Ltd.). Nitrocellulose and filter paper were pre-soaked in electroblotting transfer buffer. The gel was carefully placed onto a nitrocellulose sheet and then sandwiched between two layers of 10 filter papers and electroblotted at 15 V for 30 min.

Prevention of non-specific antibody binding was achieved by blocking nitrocellulose membranes overnight at 4 °C or 1 h at room temperature in appropriate blocking solution; typically 5 % milk-PBS-T or BSA for mAb104. Appropriate primary antibody was diluted according to Table 2.2 and incubated for 1 h at room temperature (or overnight for mAb104). Membranes were washed

three times in PBS-T for 5 min. This was followed by species-specific secondary antibody (Table 2.3) incubation for 1 h at room temperature, in the dark after which the nitrocellulose membrane was washed in twice in PBS-T for 5 min. Membranes were stored in PBS before being analysed. Immunoreactivity was detected using the LI-COR Odyssey® infrared imaging (LI-COR Biosciences, Lincoln, NE, USA) by scanning at 700 and 800 nm. Scan intensity was adjusted according to signal strength from an initial scan at default intensity.

Chapter Three

3 Tau expression in brain regions affected in Alzheimer's disease.

TDP-43 (transactive response (TAR) DNA-binding protein 43) is an RNA-binding protein involved in RNA splicing. The discovery of TDP-43 as the major constituent of pathological hallmarks present in some neurodegenerative disorders including amyotropic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U) came about in the last five years (Arai et al., 2006); Neumann et al., 2006). In these TDP-43 proteinopathies, TDP-43 is mis-localised from the nucleus to the cytoplasm forming aggregates (TDP-43 inclusions). Interestingly, 20 – 30 % of AD patients, have the characteristic TDP-43 pathology seen in FTLD-U and ALS. The TDP-43 inclusions found in these AD cases are limited to the limbic system including the amygdala and hippocampus as explained in Chapter One. TDP-43 pathology observed in these AD patients may contribute to severe clinical symptoms and neuropathology seen in these patients (Amador-Ortiz et al., 2007b; Josephs et al., 2008; Kadokura et al., 2009; Wilson et al.). However, from the pathological report documented in appendix 1.1, there was no observed pathology in the majority of the cerebellar samples used in this study.

We hypothesise that in sporadic AD cases; abnormal tau exon 10 splicing may occur and alter the 4R/3R tau ratio in regions affected in AD. It is possible that tau exon 10 inclusion is increased in AD cases similar to that observed in other tauopathies such as CBD and PSP to cause abnormal neuronal function as well as FTDP-17 where there is confirmed abnormal tau exon 10 splicing. This increased expression of tau exon 10 which will increase the 4R/3R tau ratio. TDP-43 pathology in a subset of these AD cases may either exascerbate or ameliorate this abnormal splicing. Hence, TDP-43 inclusions may be protective whilst being detrimental to the proper functioning of a neuron. This might explain the severe clinical pathologies in AD cases with TDP-43 inclusions (Amador-Ortiz et al., 2007b) in regards to tau splicing.

In this chapter, RT-PCR analysis was performed by Mr Michael Niblock to investigate the tau exon 10 splicing pattern of tau mRNA isoforms in AD cases with and without TDP-43 pathology. Also, total tau expression levels were investigated in AD patients with and without TDP-43 pathology in different brain regions of sporadic AD brain. Furthermore, we investigated whether the presence of TDP-43, influences tau alternative splicing or the total tau expression in sporadic AD. From here on AD cases with TDP43 inclusions will be referred to as ADTDP43+ and AD cases without TDP43 inclusions as ADTDP43-. For this we first performed RNA extraction of the different brain regions and analysed the tau 4R and 3R isoforms profile by RT-PCR. Secondly, we have examined the total levels of tau by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in the different AD groups; Eight controls, fifteen ADTDP43- cases and eight ADTDP43+ cases were examined. Five brain areas from each case were selected for this study. They include four areas known to be heavily affected with NFTs in AD (frontal cortex, temporal cortex, amygdala and hippocampus) and one area only slightly affected or unaffected (cerebellum). These brain regions have varying involvement of NFT pathology during the stages of disease development (Braak and Braak, 1995; Chambers et al., 1999; Yasojima et al., 1999). The cerebellum is used as a control region from affected individuals because it is largely unaffected in AD with fewer occurrences of NFTs and functional damage in AD (Braak and Braak, 1995).

For all subsequent experiments, we considered the following criteria: a) control cases analysed were void of any pathology such as tau aggregates or amyloid plaques or symptoms and b) AD cases are all sporadic cases with advanced disease (Braak and Braak stages V-VI).

3.1 Tau exon 10 alternative splicing in brain regions affected in AD.

We used RT-PCR analysis to determine tau mRNA isoforms expression in ADTDP43-, ADTDP43+ and age-matched control cases. Total RNA extracted from the frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum of ADTDP43-, ADTDP43+ cases and age-matched controls was reverse

transcribed to produce cDNA. Tau 9 – 13 primers previously designed by Dr Teresa Rodriguez Martin (King's College London) (Rodriguez-Martin et al., 2005) allowed the amplification of both 4R tau and 3R tau sequences simultaneously by PCR. The forward primer is adapted by the presence of a 5' infra-red label (IRD 700) as detailed in the methods section.

These primers are predicted to amplify a product of 367 base pairs for the cDNA containing exon 10 (4R tau) and 274 base pairs for the cDNA without exon 10 (3R tau). The intensity of tau spliced products; 4R and 3R tau bands were measured using the Li-Cor odyssey scanner and quantified using the manufacturer's software. 4R tau is quantified as mean percentage of exon 10 inclusion relative to total tau transcripts (mean percentage ± SEM). Statistical analysis was performed using Student's unpaired *t*-test.

3.1.1 Tau exon 10 splicing in frontal cortex of AD brain.

The frontal cortex, which is involved in the control of skilled movements, mood and planning, is one of the brain regions affected in the later stages of AD (Tamminga and Buchsbaum, 2004). To investigate whether the frontal cortex with NFT pathology has altered pattern of tau alternative splicing, we analysed the 4R and 3R tau expression in the frontal cortex of AD brain compared to controls.

In the frontal cortex of ADTDP43- and ADTDP43+ cases as well as age-matched controls, we found expression of two transcripts; an upper band corresponding to 4R tau and a lower band corresponding to 3R tau (Figure 3.1A).

In controls, the 4R/3R tau ratio is 1.03, similar to expected levels in normal human brain (Figure 3.1B). The 4R/3R tau ratio in ADTDP43- (1.30) and ADTDP43+ (1.46) were statistically similar to that found in age-matched controls (Figure 3.1B). Although the frontal cortex is one of the areas affected in AD with characteristic hallmarks including neurofibrillary tangles, the tau isoforms ratio is not significantly different to that observed in normal human brain regions.

3.1.2 Tau exon 10 splicing in temporal cortex of AD brain

The temporal cortex, involved in the control of hearing, memory and language functions; is also one of the regions affected in AD brain. We analysed the alternative splicing pattern of tau exon 10 in the temporal cortex of AD patients, which develops neurofibrillary tangles at the early stages of AD.

In all samples from ADTDP43- and ADTDP43+ cases and age-matched controls, tau transcripts including (4R) or excluding (3R) exon 10 were produced (Figure 3.2 A). In controls, the 4R/3R tau ratio was 0.83, which is approximately 1 as expected in normal human brain (Figure 3.2 B). The 4R/3R tau ratio in ADTDP43- was 1.40 and in ADTDP43+ was 1.22; both of which were not significantly different from that observed in compared to controls (Figure 3.2 B).

Hence, regardless of TDP-43 inclusions, AD cases did not show any difference in the 4R/3R tau alternative splicing pattern compared to age-matched individuals not suffering from AD.

3.1.3 Tau exon 10 splicing in amygdala of AD brain

The amygdala controls many brain functions including emotions, learning and memory. It also controls reflective emotions such as fear and anxiety.

We investigated the alternative splicing of tau exon 10 in the amygdala of AD brain which has characteristic neurofibrillary tangles. In all samples from ADTDP43- and ADTDP43+ cases and age-matched controls, we detected tau transcripts with both exon 10 inclusion (4R) and exon 10 exclusion (3R) (Figure 3.3 A).

In controls, the 4R/3R tau ratio was 0.85 (Figure 3.3 B). The 4R/3R tau ratio in ADTDP43- was 1.52 and was significantly increased by 78.8 % compared to controls (1.52 % ± 0.08 vs. 0.85 ± 0.09, $P<0.005$) (Figure 3.3 B). However, the 4R/3R tau ratio in ADTDP43+ was 1.50 and was not statistically different from

that observed in controls. The 4R/3R tau ratio in the amygdala was significantly increased in ADTDP43- but not in ADTDP43+ cases (Figure 3.2B).

3.1.4 Tau exon 10 splicing in the hippocampus of AD brain

One of the areas first affected in AD is the hippocampus which is involved in the formation of long term memory; as the disease progresses, pathology spreads to the surrounding lobes.
We next analysed the alternative splicing of tau exon 10 in the hippocampus of AD patients.

In all samples from ADTDP43- and ADTDP43+ cases and age-matched controls, we detected tau transcripts with exon 10 (4R) and without exon 10 (3R) (Figure 3.4 A).

In controls, the 4R/3R tau ratio was 1.15 (Figure 3.4 B). The 4R/3R tau ratio in ADTDP43- and ADTDP43+ was 2.00 and 2.11 respectively. 4R/3R tau ratio in ADTDP43- was significantly increased by 73.9 % (2.00 ± 0.26 vs. 1.15 ± 0.22, $P<0.05$) but not in ADTDP4+ (2.11 ± 0.58 vs. 1.15 ± 0.22) compared to controls (Figure 3.4 B).

3.1.5 Tau exon 10 splicing in the cerebellum of AD brain

The cerebellum is involved in the control of movement and posture. This brain region is usually unaffected in AD. The alternative splicing of tau exon 10 in the cerebellum of AD patients was next analysed.

In all samples from ADTDP43- and ADTDP43+ cases and controls, we detected tau transcripts with exon 10 (4R) and without exon 10 (3R) (Figure 3.5 A).

In controls, the 4R/3R tau ratio was 1.04 (Figure 3.5 B). The 4R/3R tau ratio in ADTDP43- and ADTDP43+ was 1.54 and 1.47 respectively. 4R/3R tau ratio in ADTDP43- was significantly increased by 48 % (1.54 ± 0.11 vs. 1.04 ± 0.17, $P<0.05$) but not in ADTDP4+ (1.47 ± 0.16 vs. 1.04 ± 0.17) compared to controls (Figure 3.5 B).

3.2 Total tau expression in brain regions affected in AD.

To gain insight into whether the expression ratios of 4R tau and 3R tau reflects a corresponding change in total tau levels in brain regions affected in sporadic AD cases, the total tau expression levels in the amygdala and hippocampus of AD patients as well as other brain regions affected in AD including the frontal cortex and temporal cortex were investigated. The cerebellum was used as a control brain region because it is largely unaffected in AD. Total RNA extracted from the frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum of fifteen ADTDP43-, eight ADTDP43+ and eight age-matched normal control subjects was analysed by RT-qPCR. For all experiments in each brain region, we considered the following criteria:

a) The control cases analysed were void of any pathology such as tau aggregates or amyloid plaques;

b) Only cases with similar expression of GAPDH and β–actin were selected for analysis because of variability between samples such as post mortem delay and RNA quality. GAPDH and β–actin house-keeping genes were used as reference genes to normalise RNA expression of total tau.

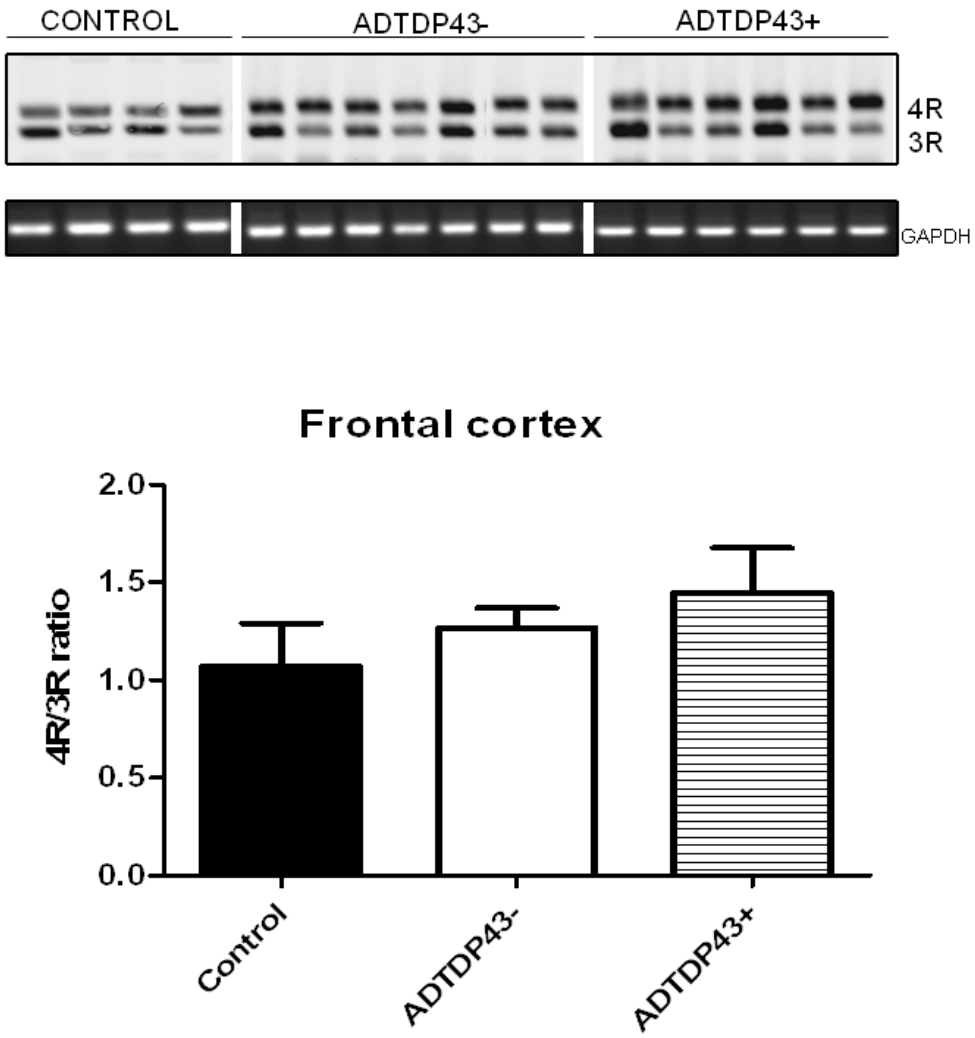


Figure 3.1. Tau exon 10 splicing in frontal cortex of Alzheimer's disease and control brain.

(A) Representative agarose gel of RT-PCR products obtained from RNA of frontal cortex from AD (ADTDP43- and ADTDP43+) brain and age-matched controls. 4R/3R tau spliced products were detected using infrared labelled primers that amplified tau between exons 9 and 13. A prominent band was detected around 367 bp corresponding to tau with 4 repeats (4R, tau) and a smaller band was detected at 274 bp corresponding to tau with 3 repeats (3R, tau) in control, ADTDP43- and ADTDP43+ cases. (B) The the 4R/3R tau ratio in controls is at 1.03. The the 4R/3R tau ratio in ADTDP43- and ADTDP43+ cases is similar to controls, showing no significant difference between the groups. Significance was set at $P<0.05$, Student's unpaired *t* test; n, control = 4, ADTDP43- = 7, ADTDP43+ = 6

RT-qPCR revealed that the total tau RNA level in the amygdala was 1.4-fold higher in ADTDP43- (2.37 ± 0.14 vs. 1.00 ± 0.62, $P < 0.05$) and 2.2-fold higher in ADTDP43+ (3.24 ± 0.14 vs. 1.00 ± 0.62, $P < 0.05$) compared to age-matched controls (Figure 3.6). However, in the frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls, there was no significant difference in the level of total tau RNA (Figure 3.6). Similarly, in the temporal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls, there was no significant difference in the level of total tau RNA expression (Figure 3.6). Likewise, there was no significant difference in of total tau RNA levels in the hippocampus and cerebellum of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.6).

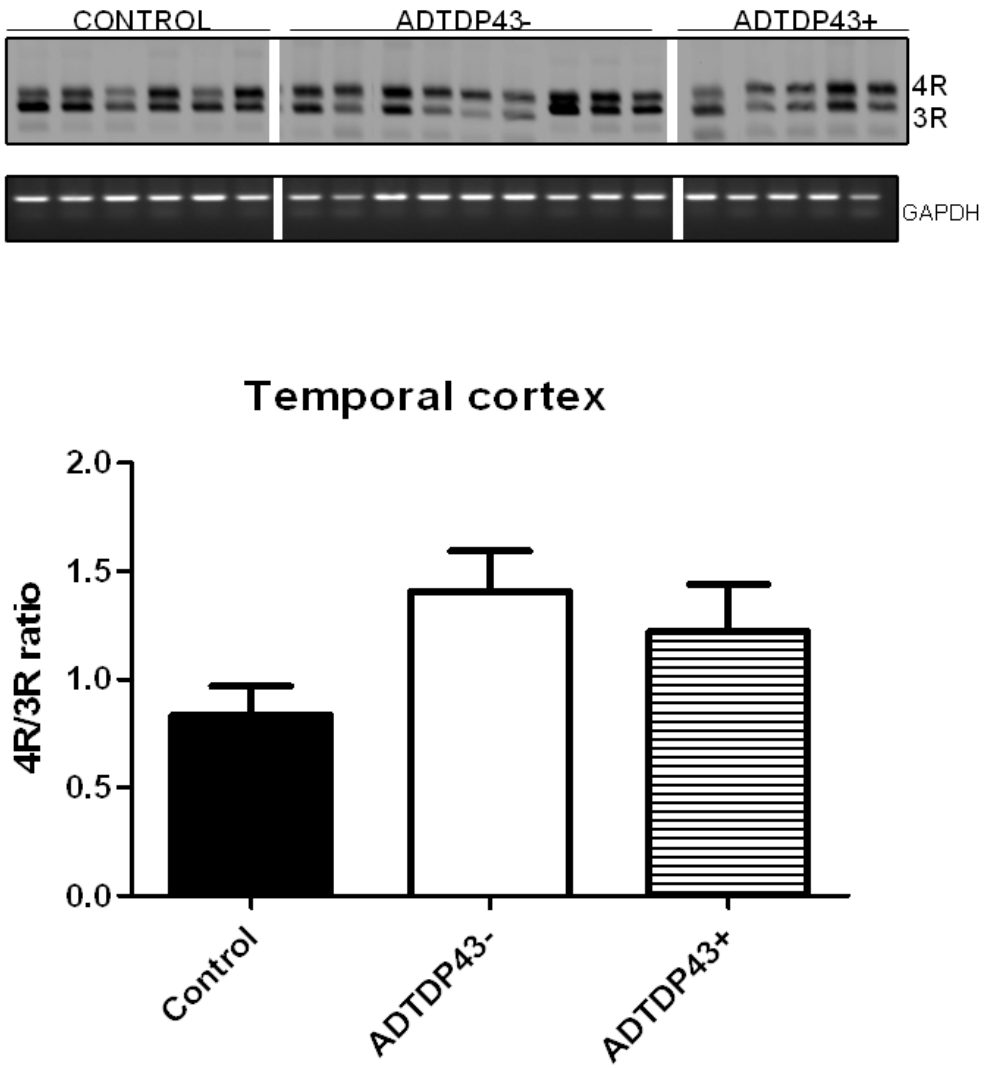
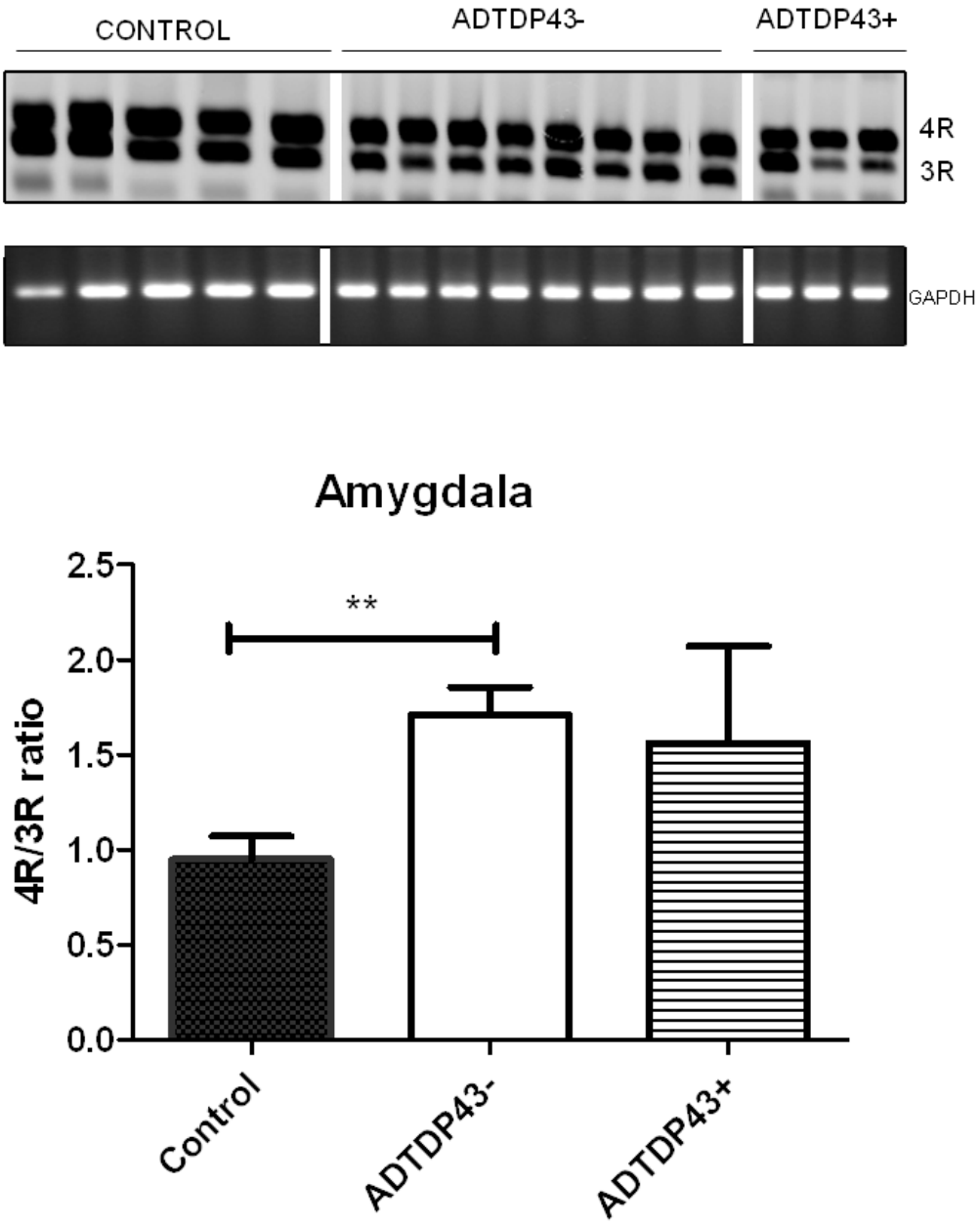
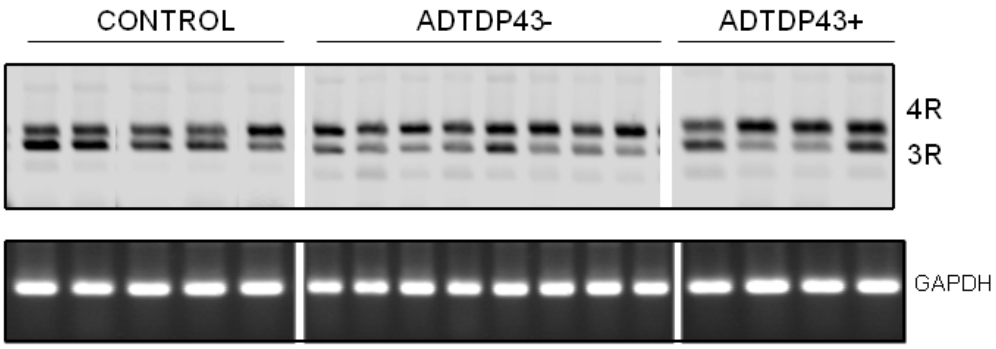


Figure 3.2. Tau exon 10 splicing in temporal cortex of control and AD brain. (A) Representative agarose gel of RT-PCR products obtained from RNA of temporal cortex from AD (ADTDP43- and ADTDP43+) brain and age-matched controls using infrared labelled primers, tau 9 - 13. (B) The 4R/3R tau ratio in ADTDP43- and ADTDP43+ cases was not statistically different from controls, showing no difference between the groups. Significance was set at $P<0.05$, Student's unpaired *t* test; n, control = 6, ADTDP43- = 9, ADTDP43+ = 5.



igure 3.3. Tau exon 10 splicing in the amygdala of control and AD brain.

(A) Representative agarose gel of RT-PCR products obtained from RNA of amygdala from AD (ADTDP43- and ADTDP43+) brain and age-matched controls using infrared labelled primers, tau 9 - 13. (B) The 4R/3R tau ratio in ADTDP43- cases is significantly increased by 78.8 % compared to controls. The 4R/3R tau ratio in ADTDP43+ cases is similar to controls, showing no significant difference between the groups. *****, $P<0.05$, Student's unpaired *t* test; n, Control = 5, ADTDP43- = 8, ADTDP43+ = 3.



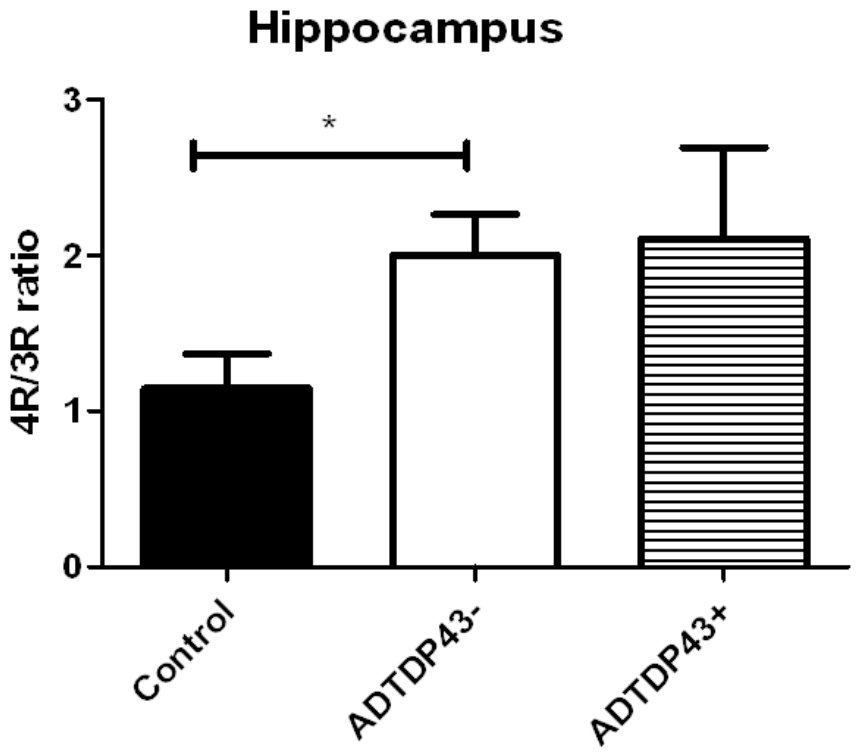
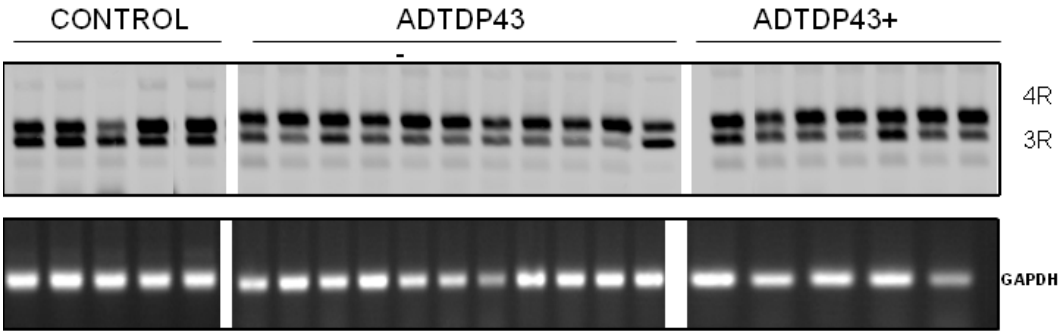


Figure 3.4. Tau exon 10 splicing in the hippocampus of control and AD brain. (A) Representative agarose gel of RT-PCR products obtained from RNA of hippocampus from AD (ADTDP43- and ADTDP43+) brain and age-matched controls using infrared labelled primers, tau 9 - 13. (B) The 4R/3R tau ratio in ADTDP43- was significantly increased by 73.9 % compared to controls. The 4R/3R tau ratio in ADTDP43+ cases is similar to controls, showing no significant difference between the groups. *****, $P<0.05$, Student's unpaired *t* test; n, control = 5, ADTDP43- = 8, ADTDP43+ = 4.



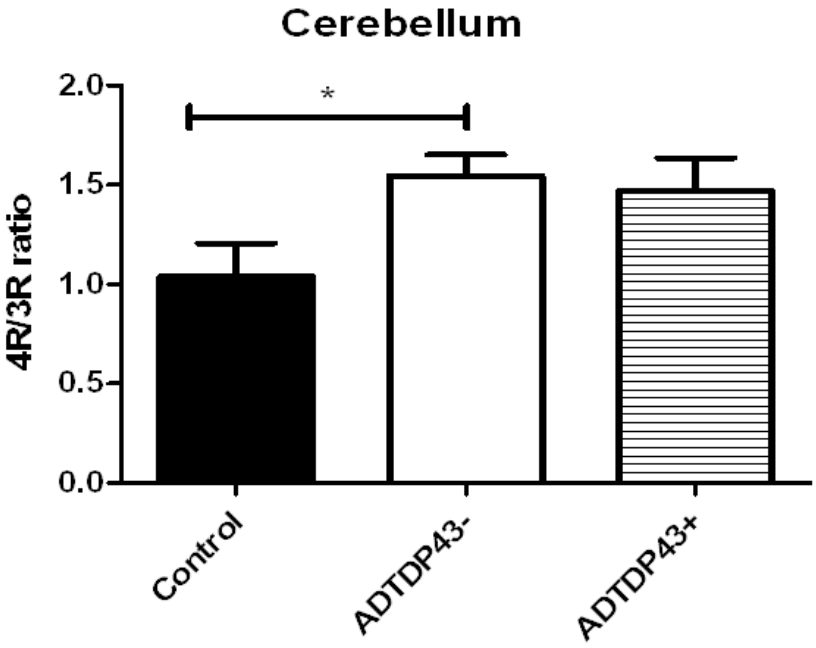


Figure 3.5. Tau exon 10 splicing in cerebellum of control and AD brain.

(A) Representative agarose gel of RT-PCR products obtained from RNA of cerebellum from AD (ADTDP43- and ADTDP43+) brain and age-matched controls using infrared labelled primers, tau 9 - 13. (B) The 4R/3R tau ratio in ADTDP43- was significantly increased by 48 % compared to controls. The 4R/3R tau ratio in ADTDP43+ cases is similar to controls, showing no significant difference between the groups. *****, $P<0.05$, Student's unpaired *t* test; n, control = 5, ADTDP43- = 11, ADTDP43+ = 7.

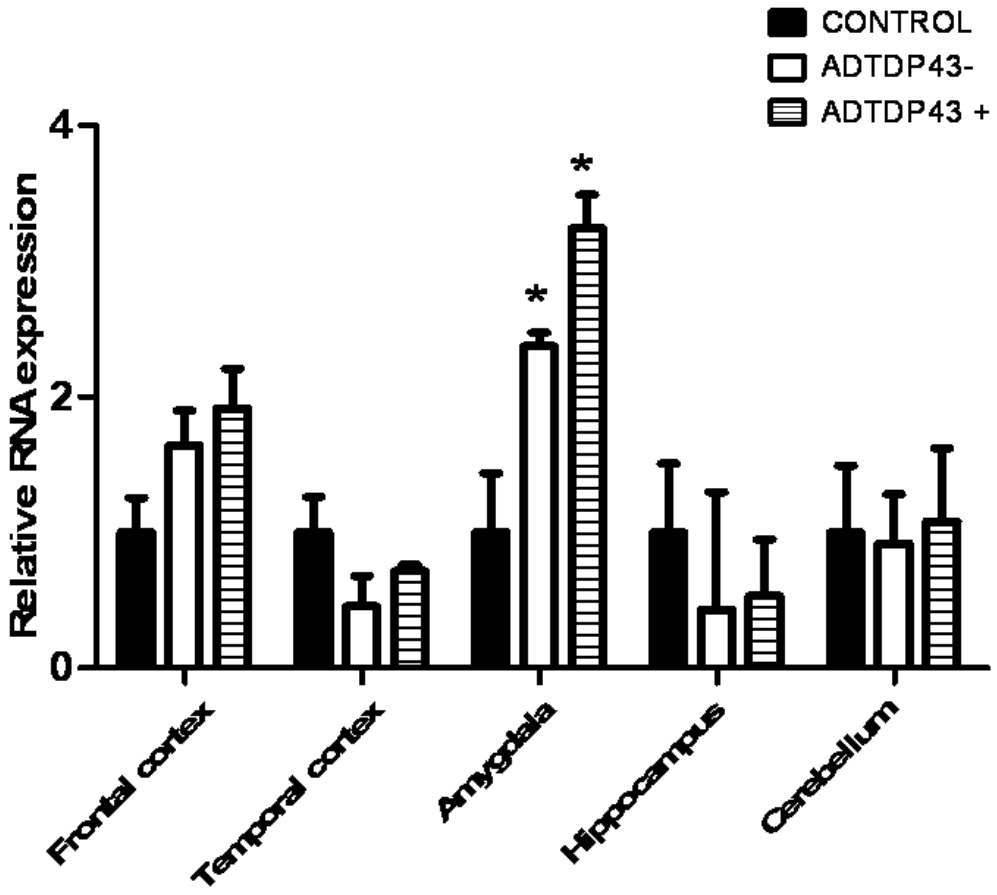


Figure 3.6. Total tau expression in control and Alzheimer's disease (AD) brain.

Total RNA was extracted from four brain regions affected in AD (frontal cortex, temporal cortex, amygdala and hippocampus) and one region largely unaffected in AD, the cerebellum. AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. In the frontal cortex temporal cortex, hippocampus and cerebellum, there was no significant change in the levels of expression of total tau in ADTDP43- and ADTDP43+ cases compared to controls. However, total tau expression was significantly increased in the amygdala of ADTDP43- and ADTDP43+ cases compared to controls. Data are normalised mean values with error bars indicating ± SEM of two independent experiments. One way ANOVA; *, $P< 0.05$. Newman-Keuls Multiple Comparison Test. n, control = 4, ADTDP43- = 6, ADTDP43+ =3.

3.3 Summary

Although an increase in the expression of 4R tau in affected regions of AD has been previously shown, this is the first time that the pattern of tau exon 10 splicing as well as total tau levels in AD cases with and without TDP-43 inclusions are examined.

The results presented in this chapter have demonstrated that:

- There is a significant increase in levels of 4R tau in selectively vulnerable brain regions affected in AD including the temporal cortex, amygdala and hippocampus.

- We found a significant increase in 4R tau levels in the temporal cortex and amygdala of ADTDP43- cases but not in ADTDP43+ cases.

- In the hippocampus, we found an increased expression of 4R tau in AD cases irrespective of TDP-43 inclusions.

- We also found an increase in 4R tau isoform in the cerebellum, a region with no NFT pathology in AD, irrespective of TDP-43 inclusions.

- The total tau levels were increased only in the amygdala of AD cases irrespective of TDP-43 inclusions.

  These findings show the tau exon 10 alternative splicing and total tau profile in brain regions affected in AD. Taken together these results reveal aberrant splicing of tau pre-mRNA in tangle-prone areas of affected AD brain. The results suggest that the increase in 4R tau and subsequently total tau levels may contribute to the formation of paired helical filaments in NFT pathology seen in sporadic AD and not caused exclusively by post-translational modifications. However the observation that changes are found in the cerebellum, a brain region relatively unaffected in AD

indicates that the changes in this brain region for tau splicing are not directly related to the degree of pathology in the AD samples. Additional work is therefore required to elucidate the relationship between alternative splicing and pathology.

Chapter four

4 SR proteins

In this chapter, we describe the expression of SR proteins that regulate tau splicing in AD patients with and without TDP-43 inclusions in different brain regions with NFTs.

We hypothesise that aberrant expression of splicing factors (SR proteins) regulating tau splicing may alter tau splicing and TDP-43 pathology may modulate the expression of splicing factors and contribute to pathogenesis of disease. SC35, SRp40 and SRp55 are focused on in this Chapter because they modulate tau exon 10 splicing *in vitro* (Kondo et al., 2004; Qian et al., 2011a; Wang et al., 2004). Since, SC35 and SRp40 are promoters of tau exon 10 inclusion *in vitro*; in brain regions where tau exon 10 splicing is abnormal (increase in 4R/3R tau ratio), SC35 and SRp40 expression may be increased while SRp55 which inhibits tau exon 10 inclusion *in vitro* may be decreased in the presence of an increased exon 10 inclusion in sporadic AD cases. SC35, SRp40 and SRp55 were the SR proteins of focus in this thesis because a lot is known about their function on tau exon 10 splicing *in vitro* (Kondo et al., 2004; Qian et al., 2011a; Wang et al., 2004).

4.1 Characterisation of mAb104

Monoclonal antibody 104 (mAb104) binds to a phosphoepitope at the RS domain of all SR proteins including SRp20, SRp30, SRp40, SRp55, and SRp75; in interphase nuclei of many vertebrates, invertebrates and plants (Manley and Tacke, 1996; Roth et al., 1990; Roth et al., 1991; Zahler et al., 1992).

To confirm the recognition of endogenous SR proteins by mAb104 antibody, immunocytochemistry was performed with HeLa cells (Figure 4.1). The pan-SR protein antibody, mAb104 was produced using hybridoma cells that secrete the antibody into the growth medium (detailed in Methods section). mAb104 binds to the phosphoepitope in the RS domain of all SR proteins. Nuclear speckles are sites for splicing factor storage in the nucleus, from where they are recruited to the spliceosome for transcription and RNA processing (Roth et al., 1991). The mAb104 detects a nuclear speckled pattern present in the nucleus of HeLa cells (Figure 4.1 A and C). We have confirmed that the mAb104 detects endogenous SR proteins localized within nuclear speckles, in agreement with previous data on the localisation of SR proteins (Caceres et al., 1997).

4.1.1 Characterisation of mAb104 with COS cells transfected with specific SR proteins

To verify the reactivity of mAb104, COS cells were transfected with DNA constructs encoding specific SR proteins: ASF2, SRp55, Tra2β, SRp40, SRp30 and SRp20. PTB, a transcriptional splicing repressor was used as a negative control to confirm the specificity of mAb104 antibody (Figure 4.2). The expression of the transfected SR proteins DNA was determined by western blot analysis of whole-cell lysates. mAb104 detects three bands at 75, 55 and 30 kDa in the non-transfected COS cells corresponding to SRp75, SRp55 and SRp30 (Figure 4.2) (Roth et al., 1991; Zahler et al., 1992). In COS cells, transfected with SRp55 and SRp40, strong bands are detected by the antibody corresponding to their size of 55 and 40 kDa respectively (Figure 4.2). The absence of any band in COS cells transfected with PTB confirmed the specificity of mAb104 for SR proteins. A 40 kDa band is also detected for the SR-related protein Tra2β (Figure 4.2).

The mAb104 antibody produced recognises exogenous SRp55, Tra2β and SRp40 in transfected COS cells but not PTB. Thus, the mAb104 antibody produced for this study recognises both endogenous and transfected individual SR proteins. Therefore, the mAb104 can be used to examine the expression of SR proteins in cell lines, rat brain and human brain tissue.

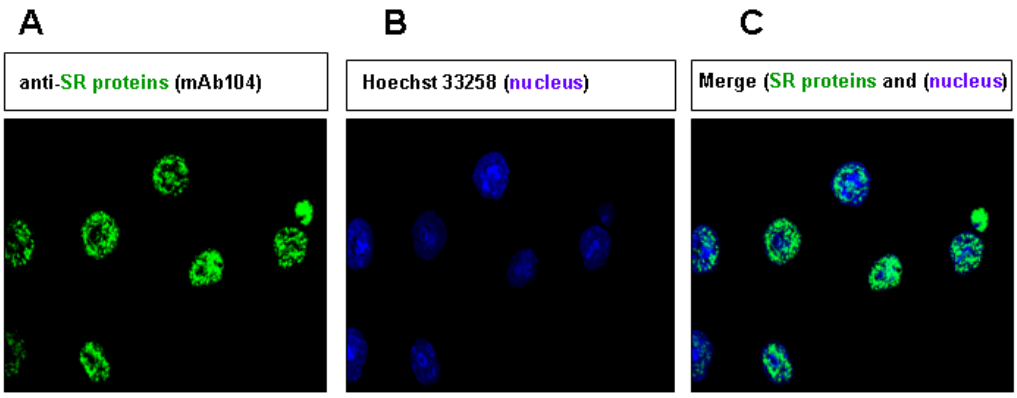


Figure 4.1. Characterisation of the anti-SR protein antibody, mAb104 in HeLa cells.

HeLa cells were fixed and stained with (A) anti-SR protein antibody, mAb104 and (B) Hoechst 33258 to visualise the nucleus; A and B are merged in (C). Cells were imaged by conventional fluorescent microscopy. mAb104 detects endogenous SR proteins in the nucleus as speckles throughout the nucleus (C).

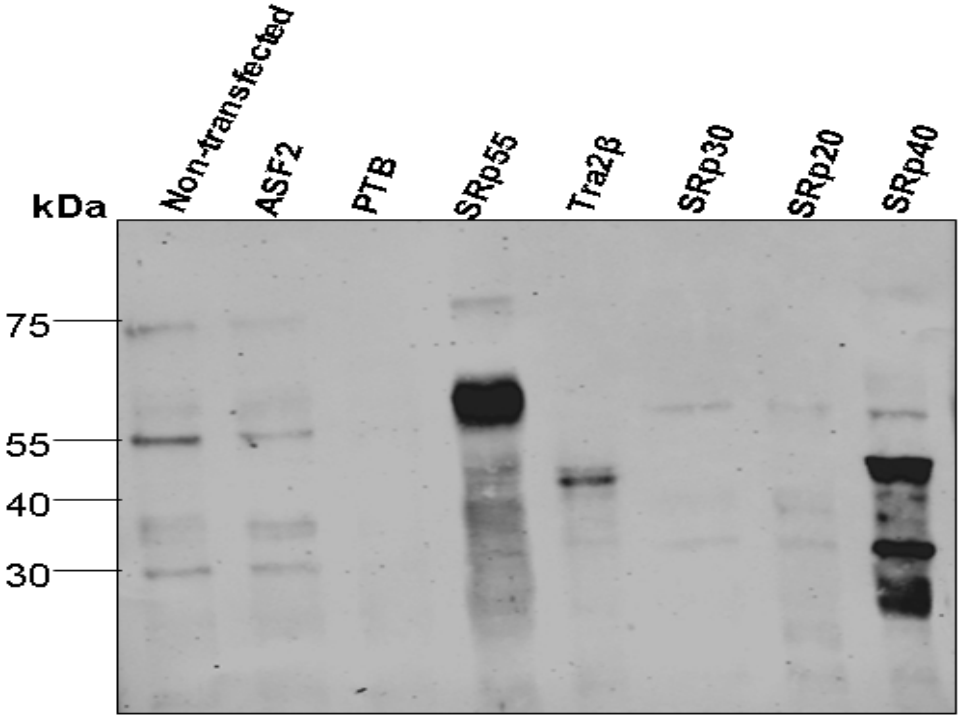


Figure 4.2. Characterisation of mAb104 using COS cells transfected with SR proteins.

COS cells were transfected with specific splicing factors: ASF2, PTB, SRp55, Tra2β, SRp30, SRp20 and SRp40; cells were harvested 24 h after transfection. Western blot analysis with mAb104 detected the presence of individual SR proteins; SRp55, Tra2β and SRp40 in transfected COS cells. SRp75, SRp55 and SRp30, corresponding to their molecular weights are expressed in the non-transfected COS cells. Molecular weight markers are indicated on the left.

4.1.2 Characterisation of mAb104 using different cell lines.

After the characterisation of the mAb104 using transfected COS cells, different cell types were used to determine the capability of mAb104 to detect endogenous SR proteins by western blot in different cell lines. Cells lysates from HeLa, COS, HEK and CHO cells were analysed by western blotting and probed with mAb104. The mAb104 detected proteins of molecular weight predicted for the different SR proteins; SRp75, SRp55, SRp40, SRp30 and SRp20 in the four cell lines (Figure 4.3). However, only COS and HEK cells show expression of SRp20 with a molecular weight of 20 kDa (Figure 4.3). Thus, we have demonstrated that the mAb104 antibody produced in this study recognises SR proteins, at their predicted sizes (although the specific identity of the individual bands requires mass spectrometric analysis) in different cell lines similar to that seen in the literature.

4.2 SR proteins expression in adult and foetal rat brain

Adult and foetal rat brain were used to validate the specificity of the mAb104 for members of the SR protein family. The expression of SR proteins in rat brain was investigated prior to the use of human brain tissue having characterised the mAb104 with transfected and non-transfected cells. SR proteins are the limiting factor in alternative splicing and may show changes in their expression levels during development (Stamm et al., 1994; Zahler et al., 1992). A gradual decline of the SR protein, SF2/ASF is observed from embryonic stages to adult rat brain (Daoud et al., 1999). Adult and foetal rat brain homogenates were prepared for western blotting analysis to detect the expression of SR proteins. Here we demonstrate that five major SR proteins are expressed in rat brain. Based on their molecular weight they are predicted to be SRp20, SRp30, SRp40, SRp55, and SRp75 with molecular weights between 20 and 75 kDa (Figure 4.4). The pattern of SR protein expression in rat brain is similar to that observed in different cell lines (Figure 4.3). However, there is an observable difference in intensity of the SR proteins between the adult and foetal brain (Figure 4.4). Although no quantitative assessment was done, extreme care was taken to ensure equal amounts of tissue was loaded by adding same volume of homogenates (5 ml 1 × sample buffer /1g of brain tissue). SR proteins at their predicted sizes (although the specific identity of the individual bands requires mass spectrometric analysis)

are readily detected in rat brain with mAb104 and a change in abundance during development from foetus to adult is plausible (Figure 4.4).

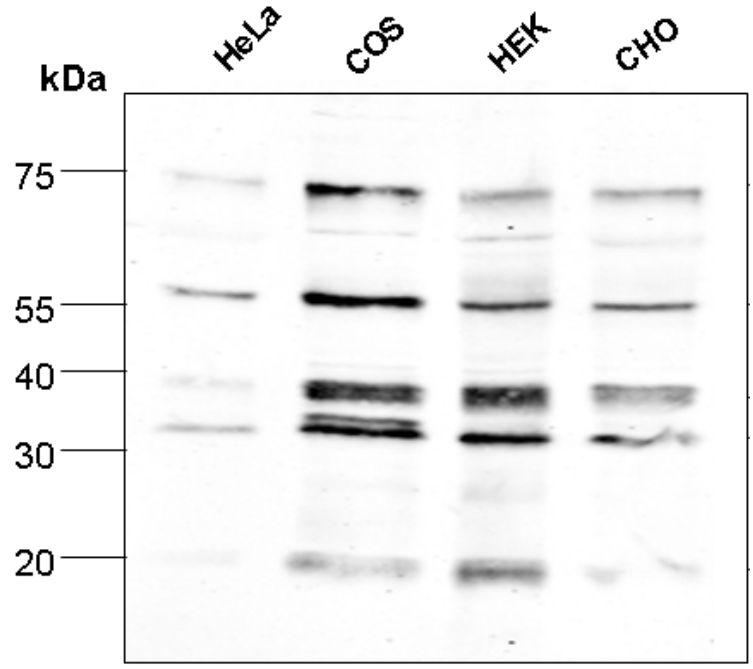


Figure 4.3. Characterisation of mAb104 in different cell lines.

Cell lysates from HeLa, COS, HEK and CHO cells were analysed by western blotting and probed with mAb104. Bands corresponding to the molecular weight of SR proteins SRp75, SRp55, SRp40, SRp30 and SRp20 are indicated. Molecular weight markers are indicated on the left.

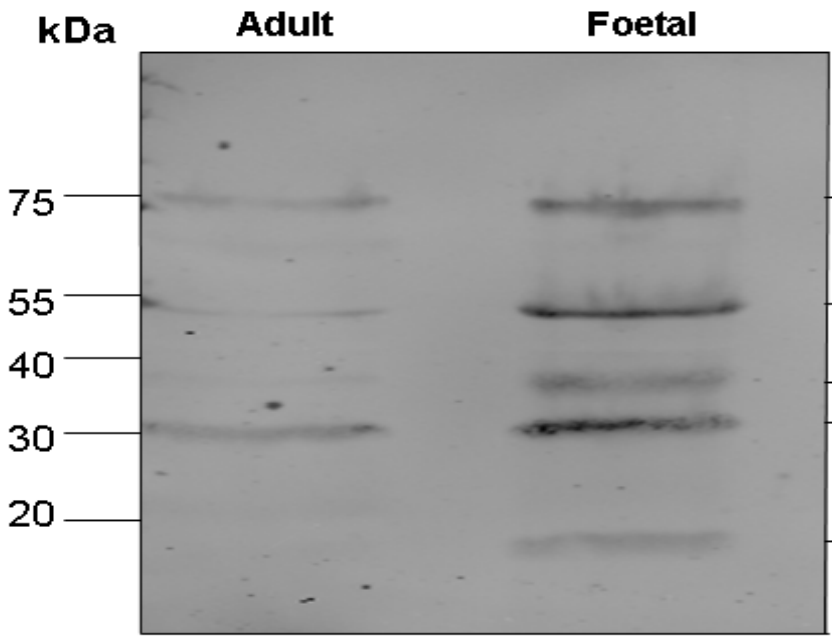


Figure 4.4. SR proteins expression in adult and foetal rat brain using mAb104 antibody.

Western blot analysis of adult and foetal rat brain homogenates were probed with mAb104. SR proteins including bands corresponding to SRp75, SRp55, SRp40, SRp30 and SRp20 are observed in both adult and foetal rat brain. Molecular weight markers are indicated on the left.

4.3 SR proteins expression in human brain

After the successful detection of SR proteins in rat brain, we analysed the expression of SR proteins in normal human brain. Total brain homogenates from three normal temporal cortices were analysed by western blotting and probed with the mAb104. The antibody detected numerous proteins of molecular weights between 30 and 75 kDa. We found that independently of age, post-mortem delay and sex, four major bands were detected by mAb104 in all three normal brain samples tentatively identified as SRp75, SRp55, SRp40 and SRp30 (Figure 4.5). While the "SRp75" and "SRp55" bands were conserved in all three normal brains. The densities of the other bands vary between the samples although equal amounts of protein were loaded seen from the level of GAPDH (Figure 4.5). The mAb104 also detects additional, unexpected bands in the three normal human brains that are not found in cell-lines or rodent samples. These may represent other members of the SR protein family for example, 9G8 with a molecular weight size of 36 kDa (Huang and Steitz, 2001), or may represent non-specific binding of antibody under these conditions.

Therefore the pattern of bands seen with is antibody in human brain, is more complex than that seen in cell lines (Figure 4.3) and in rat brain (Figure 4.4), although SR proteins are conserved from rodents to humans (Figure 4.5) (Roth et al., 1991). The specific identity of the individual bands identified in the normal human brain samples were not identified in this present study, and may include non-specific binding. Therefore the mAb104 antibody is not suitable for detailed analysis of SR protein expression in sporadic AD cases, and is means the human tissue western blot analysis results cannot be unequivocally interpreted. The specific identity of the individual bands in the normal human brain samples requires further analysis, with additional antibodies; the mAb104 antibody is not suitable for detailed analysis of predicted SR protein expression in sporadic AD cases at this time.

4.3.1 SR protein transcripts expression by RT-PCR in human brain.

After we confirmed the expression of SR proteins in normal human brains using mAb104 at the protein level, we next sought to investigate the expression of individual SR proteins at the RNA level by RT-PCR. Primers that detect each human SR protein transcript including SC35, SRp40 and SRp55 were designed (Table 2.1).

Total RNA extracted from three normal brain temporal cortices was reverse transcribed and amplified by PCR detailed in the Methods chapter. SC35, SRp40 and SRp55 expression was assayed by RT-PCR and PCR products visualised using ethidium bromide staining (Figure 4.6). RT-PCR analyses showed that SR protein members; SC35, SRp40 and SRp55 are expressed in normal brain temporal cortices at RNA level (Figure 4.6) and plausibly translated to bona fide proteins of molecular weight sizes of 30, 40 and 55 kDa detected at protein level (Figure 4.5). These specific SR protein primers are used to determine the expression levels of relevant SR proteins by RT-qPCR in brain regions affected in AD in the subsequent sections.

4.4 SR proteins expression in the regions affected in AD brain

Here, we investigate the expression levels of SR proteins including SC35, SRp40 and SRp55; in the amygdala and hippocampus of AD patients as well as other brain regions affected in AD including the frontal cortex and temporal cortex. The cerebellum was used as a control brain region because it is largely unaffected in AD. Total RNA extracted from the frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum of fifteen AD cases without TDP-43 inclusions (ADTDP43-), eight AD cases with TDP-43 inclusions (ADTDP43+) and eight age-matched normal control subjects was analysed by quantitative RT-PCR (RT-qPCR) explained in the Methods chapter.
For all subsequent experiments, we considered the following criteria:

a) Control samples analysed were void of any pathology seen in AD brain such as tau aggregates or amyloid plaques.

b) GAPDH and β–actin house-keeping genes were used as reference genes to normalise RNA expression of SR proteins; only cases with similar expression of GAPDH and β–actin were selected for analysis because of variability between samples such as post mortem delay and RNA quality. Samples with a Cycle threshold (Ct) value of more than 28 were excluded from the data analysis. Data are normalized means with error bars indicating ± SEM of two independent experiments.

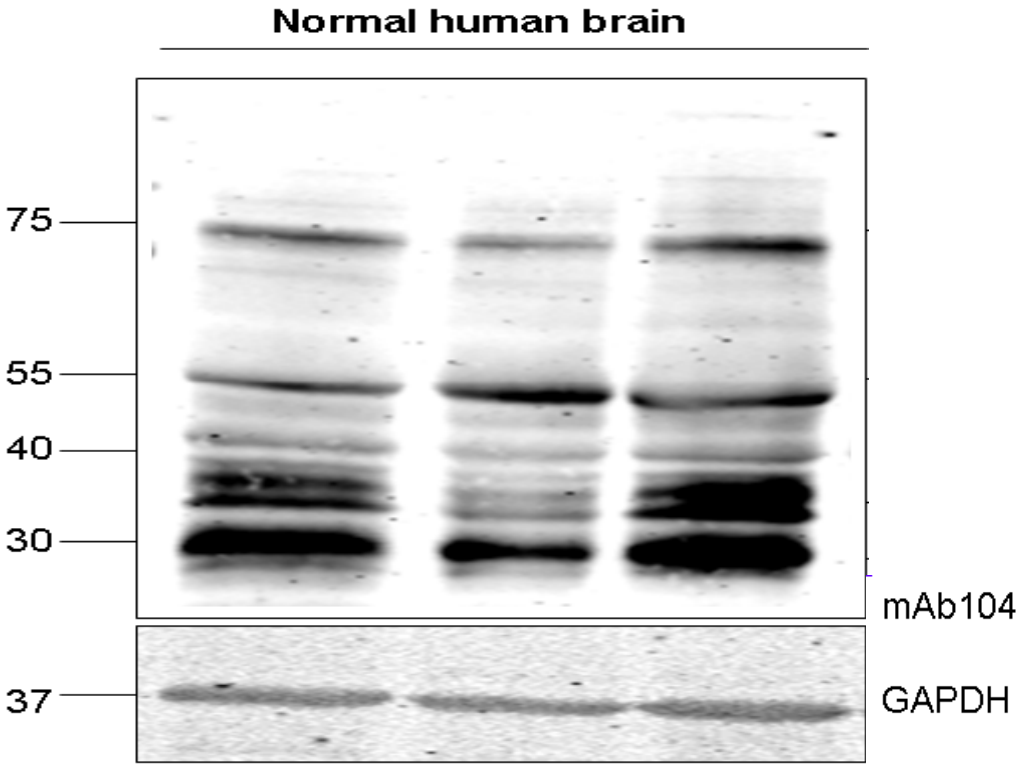


Figure 4.5. SR proteins expression in normal human brain.

Total brain homogenates from three normal brain temporal cortices were analysed by western blotting and probed with the mAb104 antibody. SR proteins of varying sizes were detected in human brain with sizes between 30 – 75 kDa. Four major SR proteins are expressed in the normal human brain, including: SRp75, SRp55, SRp40 and SRp30; indicated on the right. GAPDH (37 kDa) was used as a loading control. Molecular weight markers are indicated on the left.

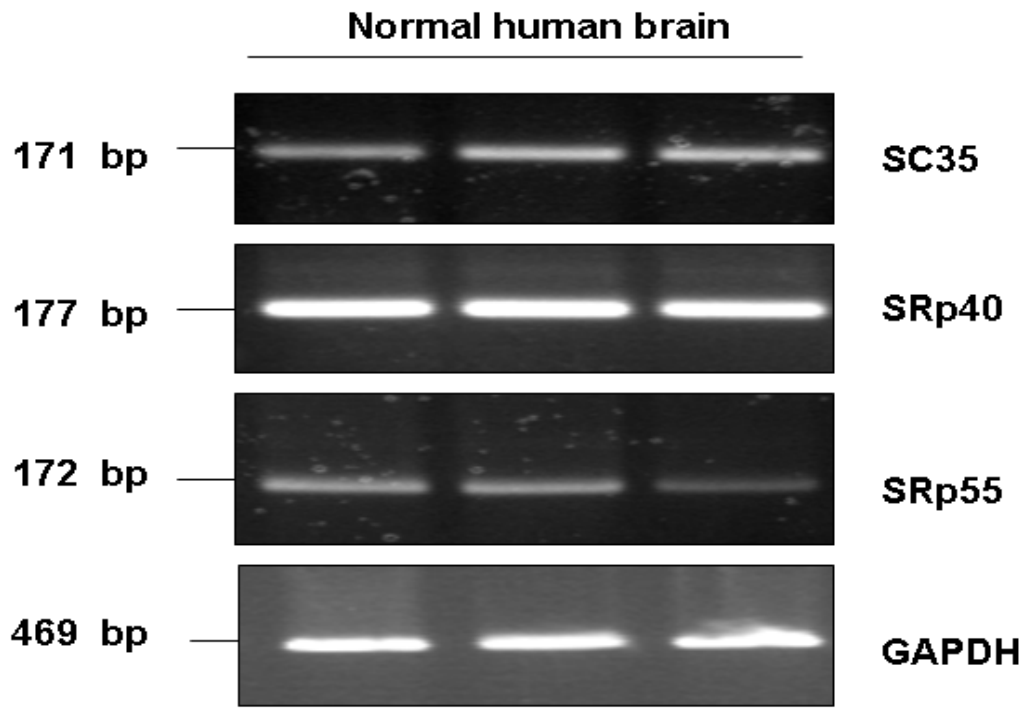


Figure 4.6. Detection of SR protein transcripts in normal human brain.
The expression of SR proteins SC35, SRp40 and SRp55 was assayed by RT-PCR using three primers specific for each human SR protein transcript. GAPDH was used as a loading control. PCR products were visualised using ethidium bromide staining. Amplicon sizes of 171 bp, 177 bp, 172 bp and 469 bp were detected for SC35, SRp40, SRp55 and GAPDH respectively.

4.4.1 SR protein expression in the amygdala of AD brain

The amygdala controls many brain functions including emotions, learning and memory. It also controls reflective emotions such as fear and anxiety. In the previous chapter, we showed that there was an increased expression of 4R tau in the amygdala of AD cases without TDP43 inclusions (ADTDP43-) (Figure 3.3).

We analysed the expression levels of SC35, SRp40 and SRp55 in the amygdala of ADTDP43-, ADTDP43+ and age-matched controls with the same primers that detected their expression in normal human brain (Figure 4.6).
Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the amygdala; these include: Control (C) cases, C1 and C4 (RIN nos < 5); ADTDP43- (A) cases A2, A8, A11, A12, A13 and A15 (RIN nos < 5); A3 and A5 (Ct value > 28) while A12 and A14 (no tissue available); ADTDP43+ (T) cases T3, T4, T6 (RIN nos < 5) while T2 and T9 (Ct value > 28).

RT-qPCR revealed that the expression levels of SC35 RNA was not different in the amygdala of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 4.7). We found a 4-fold increase in the expression SRp55 RNA only in ADTDP43- cases compared to controls, (4.91 ± 0.0234 vs. 1.00 ± 0.82, $P < 0.05$) (Figure 4.7). The expression of SRp55 RNA in ADTDP43+ cases was not significantly different to that observed in controls (Figure 4.7). Interestingly, we found a 9-fold increase in SRp40 RNA expression in ADTDP43- cases compared to normal control subjects (10.11 ± 0.24 vs. 1.00 ± 0.84, $P < 0.001$). However, there was no significant difference in the expression of SRp40 RNA in ADTDP43+ cases compared to controls (Figure 4.7).

4.4.2 SR protein expression in the frontal cortex of AD brain

The frontal cortex, which is involved in the control of skilled movements, mood and planning, is one of the brain regions affected in the later stages of AD (Tamminga and Buchsbaum, 2004).

We did not find a change in tau exon 10 expression in the frontal cortex of AD cases with and without TDP43 inclusions. We analysed the expression levels of SC35, SRp40 and SRp55 in the frontal cortex of ADTDP43-, ADTDP43+ and age-matched controls with the same primers that detected their expression in normal human brain (Figure 4.6). Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the frontal cortex; these include: Control (C) cases, C1 and C7 (RIN nos < 5); ADTDP43- (A) cases A7 and A12 (RIN nos < 5); A6, A8, A10, A11, A13, A14 and A15 (Ct value > 28); ADTDP43+ (T) cases T3 and T4 (RIN nos < 5) while T5, T8 and T9 (Ct value > 28).

RT-qPCR revealed that SC35 RNA level of expression was not different in the frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 4.8). There was no significant difference in the expression level of SRp40 RNA compared to age-matched controls (Figure 4.8). Similarly, the expression of SRp55 RNA was significantly different in ADTDP43- cases compared to age-matched controls (Figure 4.8). Furthermore, SRp55 RNA expression in ADTDP43+ cases showed no significant change compared to age-matched controls (Figure 4.8).

4.4.3 SR protein expression in the temporal cortex of AD brain

The temporal cortex, involved in the control of hearing, memory and language functions; is also one of the regions affected in AD brain. In Chapter three, we showed that there was an increased expression of tau containing exon 10 (4R) in the temporal cortex of AD cases without TDP43 inclusions (ADTDP43-) (Figure 3.2). Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the temporal cortex; these include: Control (C)

cases, C1 and C7 (RIN nos < 5); C6 (Ct value > 28); ADTDP43- (A) cases A7 and A13 (RIN nos < 5); A3, A4, A6, A9, A11, A14 and A15 (Ct value > 28); ADTDP43+ (T) cases T3, T4 and T5 (RIN nos < 5) while T8 (Ct value > 28).

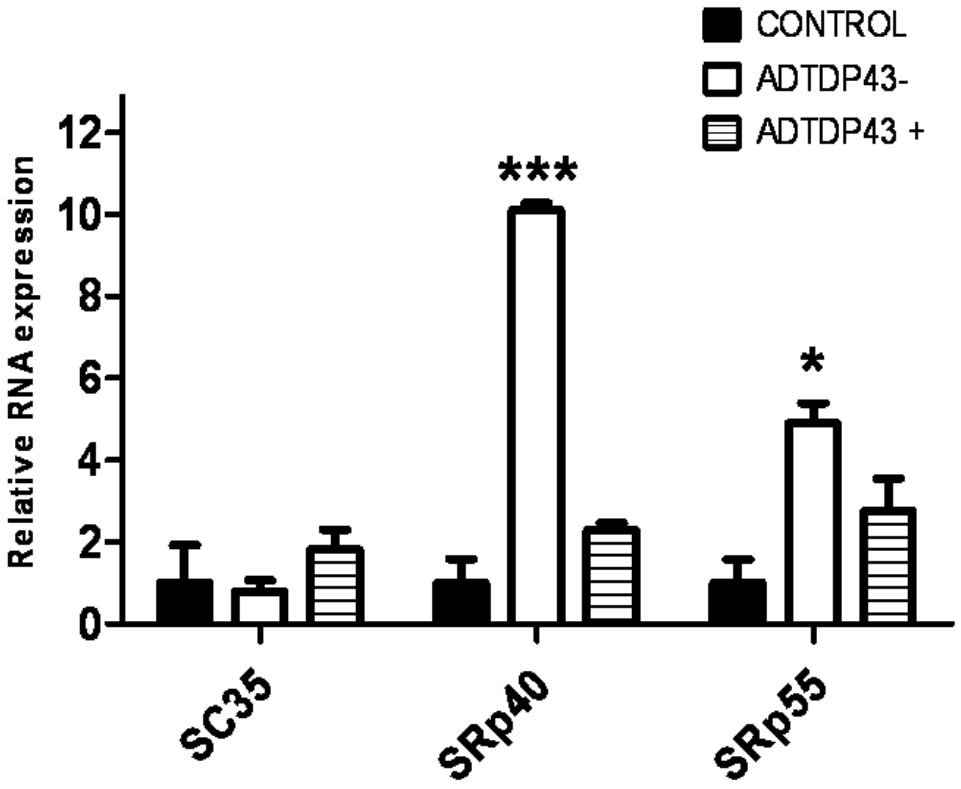


Figure 4.7. SR proteins expression in the amygdala of Alzheimer's disease brain.

SR proteins expression in the amygdala of AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. SR protein (SC35, SRp40 and SRp55) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of SC35 RNA in ADTDP43- and ADTDP43+ cases compared to controls. However, SRp55 RNA expression was significantly increased only in ADTDP43- cases compared to controls. Compared to controls, the expression of SRp40 RNA in the amygdala of ADTDP43- cases was significantly increased. One-way ANOVA *, $P<0.05$; ***, $P<0.001$ (Tukey's test). n, control = 6, ADTDP43- = 5, ADTDP43+ = 3.

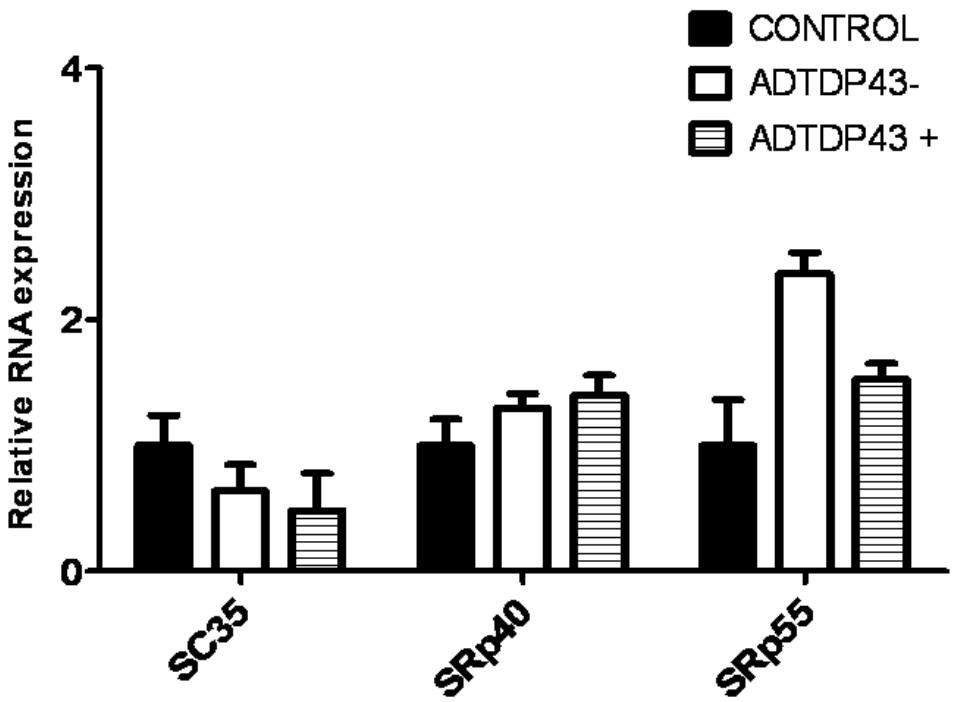


Figure 4.8. SR protein expression in the frontal cortex of Alzheimer's disease brain.

SR proteins expression in the frontal cortex of AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. SR protein expression was assayed by quantitative RT-PCR and normalised to GAPDH and β-actin. SC35, SRp40 and SRp55 expression did not show any significant changes in ADTDP43- and ADTDP43+ cases compared to controls. Significance was set at $P<0.05$, One-way ANOVA (Tukey's test); n, control = 6, ADTDP43- = 5, ADTDP43+ = 3.

Using the same primers that detect their expression in normal brain (Figure 4.6), we analysed the expression levels of SC35, SRp40 and SRp55 in the temporal cortex of ADTDP43+, ADTDP43- and age-matched controls. In the temporal cortex of ADTDP43- and ADTDP43+ cases there was no significant difference in the expression levels of SC35 and SRp55 RNA compared to age-matched controls (Figure 4.9). Likewise, we did not find any difference in the expression of SRp40 RNA in ADTDP43- cases compared to controls (Figure 4.9).

4.4.4 SR protein expression in the hippocampus of AD brain

One of the brain areas first affected in AD is the hippocampus which is involved in the formation of long term memory; as the disease progresses, pathology spreads to other lobes. In Chapter three, we showed that there was an increased expression of tau containing exon 10 (4R) in the hippocampus of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.4).

We analysed the expression levels of SC35, SRp40 and SRp55 RNA in the hippocampus of ADTDP43-, ADTDP43+ and age-matched controls with the same primers that detected their expression in normal human brain (Figure 4.6). Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the hippocampus; these include: Control (C) cases, C1, C4 and C7 (RIN nos < 5); ADTDP43- (A) cases A1, A2, A4, A7, A10, A11 and A14 (RIN nos < 5);ADTDP43+ (T) cases T3, T4, T6 and T8 (RIN nos < 5).

RT-qPCR revealed that in the hippocampus of ADTDP43- and ADTDP43+ cases there was no significant difference in the expression levels of SC35 and SRp55 RNA compared to age-matched controls (Figure 4.10). Similarly, the expression of SRp40 RNA was not different in ADTDP43- and ADTDP43+ cases compared to controls (Figure 4.10).

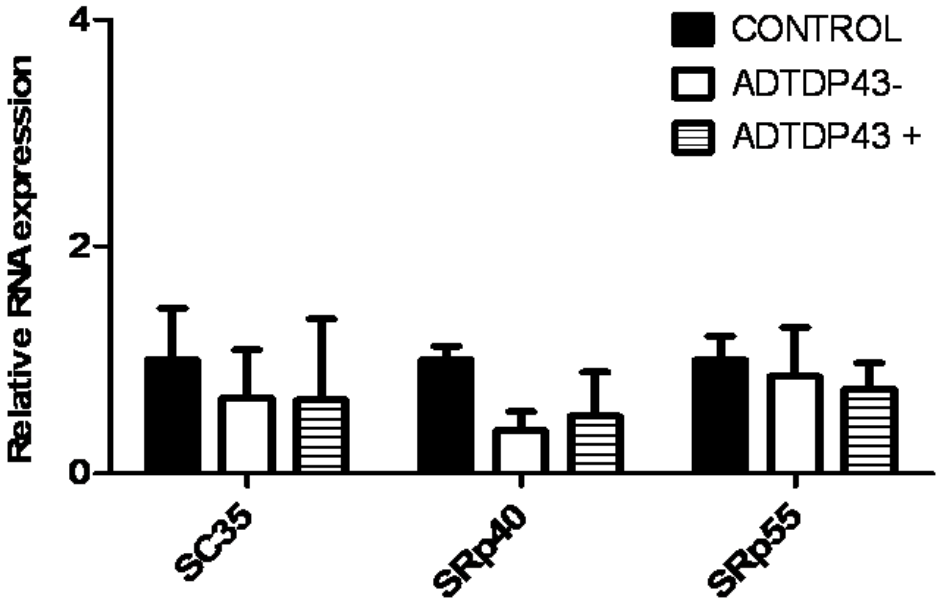


Figure 4.9. SR proteins expression in the temporal cortex of Alzheimer's disease brain.

SR proteins expression in the temporal cortex of AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. SR proteins expression was assayed by quantitative RT-PCR and normalised to β-actin and GAPDH. The expression of SC35, SRp40 and SRp55 in the temporal cortex did not show any significant changes in ADTDP43- and ADTDP43+ cases, compared to controls. Significance was set at $P<0.05$ One way ANOVA. n, control = 5, ADTDP43- = 6, ADTDP43+ =4.

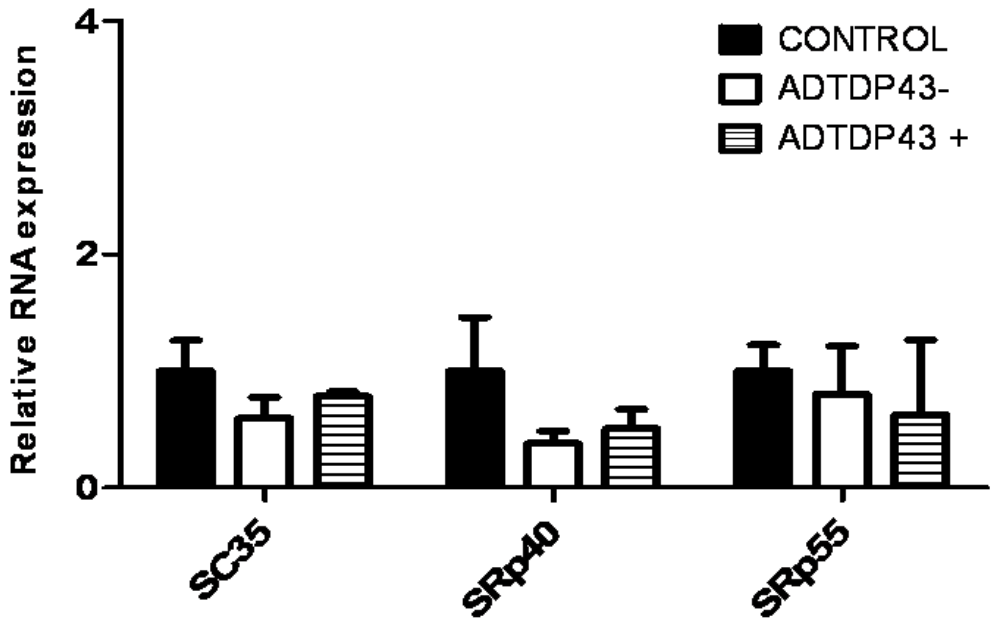


Figure 4.10. SR proteins expression in the hippocampus of Alzheimer's disease brain.

SR proteins expression in the hippocampus of AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. SR proteins expression was assayed by quantitative RT-PCR and normalised to β-actin and GAPDH. The expression of SC35, SRp40 and SRp55 in the hippocampus did not show any changes in ADTDP43- and ADTDP43+ cases, compared to controls. Significance was set at $P<0.05$ One way ANOVA. n, control = 5, ADTDP43- = 6, ADTDP43+ =4

4.4.5 SR protein expression in the cerebellum of AD brain

The cerebellum is involved in the control of movement and posture and is mostly unaffected in AD; hence, it was used as the control brain region in this study. The expression levels of SC35, SRp40 and SRp55 was next investigated.

We analysed the expression levels of SC35, SRp40 and SRp55 in the cerebellum of ADTDP43-, ADTDP43+ and age-matched controls with the same primers that detected their expression in normal human brain (Figure 4.6) by RT-qPCR. Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the cerebellum; these include: Control (C) cases, C1, C6 and C7 (RIN nos < 5); ADTDP43- (A) cases A8 (RIN nos < 5); A1, A4, A7, A9, A12 and A13 (Ct value > 28); ADTDP43+ (T) cases T3 (RIN nos < 5), T4 and T7 (Ct value > 28).

RT-qPCR revealed that in the cerebellum of ADTDP43- and ADTDP43+ cases there was no significant difference in the expression levels of SC35, SRp40 and SRp55 RNA compared to age-matched controls (Figure 4.11).

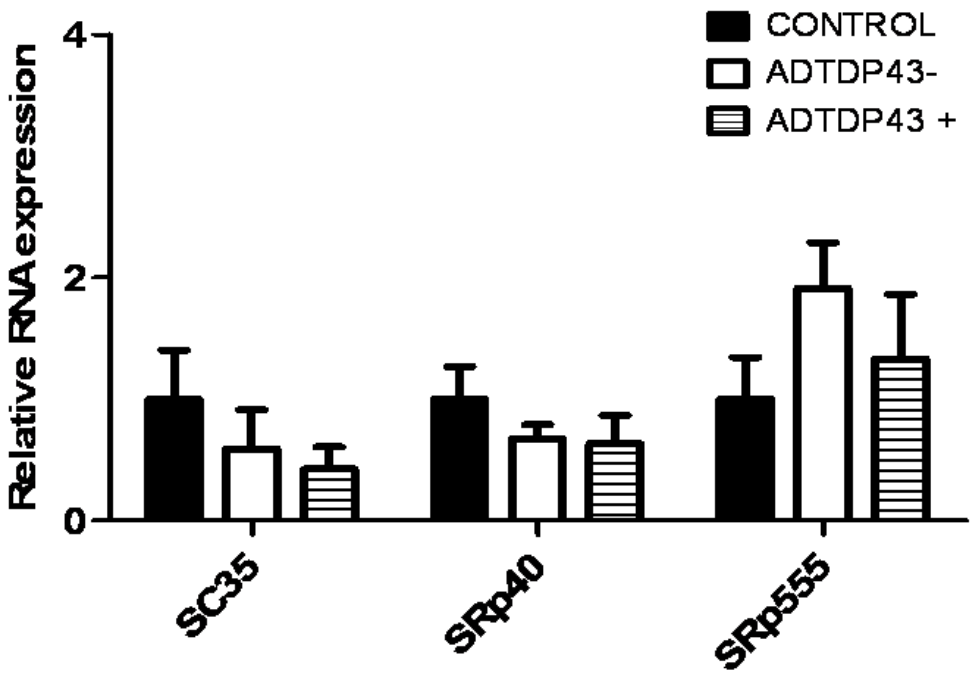


Figure 4.11. SR proteins expression in the cerebellum of Alzheimer's disease brain.

SR proteins expression in the cerebellum of AD brain with (ADTDP43 +) and without (ADTDP43 -) TDP-43 inclusions and age-matched controls were analysed. SR proteins expression was assayed by quantitative RT-PCR and normalised to β-actin and GAPDH. The expression of SC35, SRp40 and SRp55 in the cerebellum did not show any significant changes in ADTDP43- and ADTDP43+ cases, compared to controls. Significance was set at $P<0.05$ One way ANOVA. n, control = 5, ADTDP43- = 6, ADTDP43+ = 5.

A summary of the RT-qPCR analysis for SC35, SRp40 and SRp55 is shown in Figure 4.12. RT-qPCR showed that the expression level of SC35 was not different in all five brain regions of ADTDP43- and ADTDP43+ cases compared to age-matched controls. However, SRp55 expression was significantly increased only in the amygdala of ADTDP43- cases compared to controls. There was no significant difference in the expression of SRp55 in ADTDP43- and ADTDP43+ cases compared to controls in the frontal cortex, temporal cortex, hippocampus and cerebellum. SRp40 showed a significant increase in expression in the amygdala of ADTDP43- cases compared to controls. Nevertheless, there was no significant differece in the expression of SRp40 in the frontal cortex, temporal cortex, hippocampus and cerebellum of ADTDP43- and ADTDP43+ cases compared to controls.

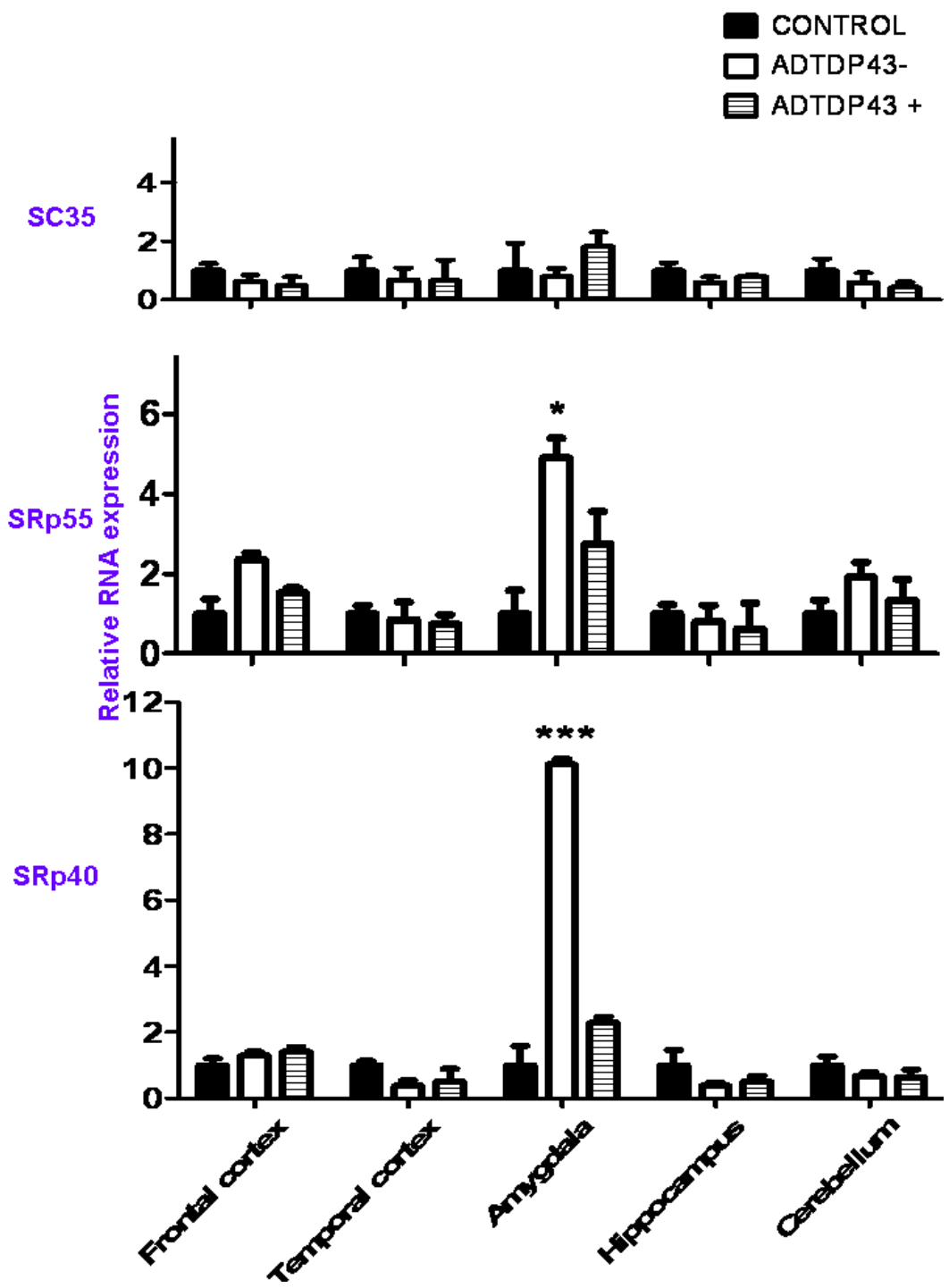


Figure 4.12. SR proteins expression in brain regions of AD brain.

SR proteins expression in AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions of four brain regions affected in AD (frontal cortex, temporal cortex, amygdala and hippocampus) and one region largely unaffected in AD, the cerebellum; was assayed by quantitative RT-PCR and normalised to GAPDH and β-actin. Data are normalised mean values with error bars indicating ± SEM. One-way ANOVA *, $P<0.05$; ***, $P<0.001$ (Tukey's test)

4.5 Summary

The results presented in this chapter have demonstrated that:

- SR protein transcripts implicated in tau exon 10 splicing regulation including; SC35, SRp40, and SRp55 are expressed at the RNA level in normal human brain. Expression of these SR proteins do not correlate with pathology in AD.

- The mAb104 antibody was unsuitable for detection of SR proteins in human brain.

- The mRNA encoding SR proteins SRp40 and SRp55 are increased in the amygdala of AD cases without TDP-43 inclusions (ADTDP43-). The increased expression of SRp40 in the amygdala of ADTDP43- cases compared to controls but not in ADTDP43+ cases suggest that in the presence of TDP-43 inclusions, SRp40 does not change.

Taken together, these findings show the expression profile of mRNAs encoding SR proteins in normal human brain as well as in brain regions affected in AD. The increase in expression of SRp40 and SRp55 mRNAs only in the amygdala of ADTDP-43- confirms our hypothesis that splicing factors can be misregulated in sporadic AD brain. However, since the amygdala is a brain region which is not the most affected in AD, we note that the change in SRp40 and SRp55 does not correlate with extent of pathology

Chapter Five

5 CELF proteins

In this chapter, we demonstrate the expression levels of CELF proteins that regulate tau exon 10 splicing in AD patients with and without TDP43 inclusions; particularly in different parts of the brain with NFT pathology.

We hypothesise that aberrant expression of splicing factors (CELF proteins) regulating tau splicing may alter tau splicing and TDP-43 pathology may modulate the expression of splicing factors and contribute to pathogenesis of disease. CELF1, CELF3 and CELF4 are focused on in this Chapter because they modulate tau exon 10 splicing *in vitro* (Chapple et al., 2007; Wang et al., 2004) Since, CELF3 and CELF4 are promoters of tau exon 10 inclusion *in vitro*; in brain regions where tau exon 10 splicing is abnormal (increase in 4R/3R tau ratio in the amygdale and hippocampus of ADTDP43- cases); it is likely that CELF3 and CELF4 expression will be increased in sporadic AD cases.

5.1 Characterisation of 3B1

To confirm that the anti-CELF1 protein antibody, 3B1 monoclonal antibody recognises endogenous CELF proteins, immunocytochemistry was performed with HeLa cells (Jiang et al., 2004; Timchenko et al., 1996).

The 3B1 antibody recognises a conserved epitope at the C-terminus RNA recognition motif (RRM3) in all members of the CELF family of proteins (Good et al., 2000).
CELF proteins are important during post-transcriptional events such as mRNA stability, alternative splicing, and translational control (Jiang et al., 2004; Ladd et al., 2001). We show that the 3B1 antibody detects CELF proteins localised diffusely in the cytoplasm and nucleus (Figure 5.1A and C); consistent with their involvement as multifunctional proteins.

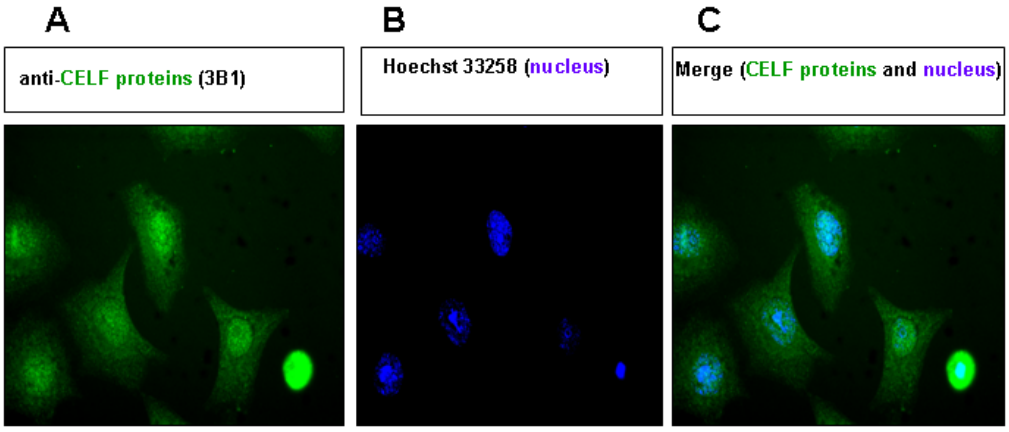


Figure 5.1. Characterisation of the anti-CELF antibody, 3B1 using in HeLa cells.

HeLa cells were fixed and stained with (A) 3B1 antibody and (B) Hoechst 33258 to visualise the nucleus; A and B are merged in (C). Cells were imaged by conventional fluorescent microscopy. CELF proteins are localised in the nucleus as well as in the cytoplasm.

5.1.1 Characterisation of 3B1 using different cell lines

After the characterisation of anti-CELF antibody, 3B1 using HeLa cells; cell lysates from HeLa and SH-SY5Y cells were analysed by western blotting and probed with 3B1 to confirm the capacity of 3B1 to detect endogenous CELF proteins in the different cell lines. The 3B1 antibody detected CELF proteins as doublet bands with molecular weight predicted for CELF proteins at 50 kDa in the two cell lines (Figure 5.2). We have demonstrated that the 3B1 antibody recognises CELF proteins in the two cell lines similar to that seen in the literature (Ladd et al., 2001).

5.2 CELF proteins expression in adult and foetal rat brain

The expression of CELF proteins is developmentally regulated in the brain (Ladd et al., 2001). We therefore wanted to determine the developmental profile of CELF proteins in rat brain after characterisation of the 3B1 antibody with HeLa and SH-SY5Y cells. This would further characterise the ability of the 3B1 antibody to detect CELF proteins in rodents although mouse brain could have been used.

Adult and foetal rat brain homogenates were prepared for western blotting analysis to detect the expression of CELF proteins. Here, we show that CELF proteins are expressed in rat brain based on their molecular weight at 50 kDa with 3B1 antibody (Figure 5.3). Interestingly, we observe a difference in intensity of CELF proteins between adult and foetal rat brain (Figure 5.3), similar to that seen with the expression of SR proteins between adult and foetal rat brain (Figure 4.4). Although no quantitative assessment was done, extreme care was taken to ensure equal amounts of tissue was loaded by adding same volume of homogenates (5 ml 1 × sample buffer /1g of brain tissue). CELF proteins are readily detected in rat brain with 3B1.

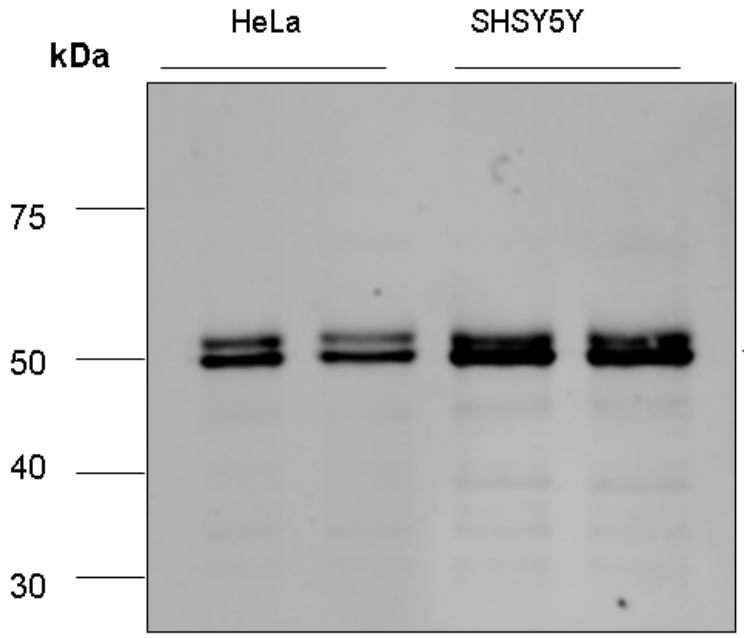


Figure 5.2. Characterisation of the anti-CELF antibody, 3B1 in different cells.

Total cell lysates extracted from HeLa and SH-SY5Y cells were analysed by western blotting and probed with the 3B1 antibody. The CELF proteins detected in both HeLa and SH-SY5Y cells appear as doublets at 50kDa. Molecular weight markers are indicated on the left.

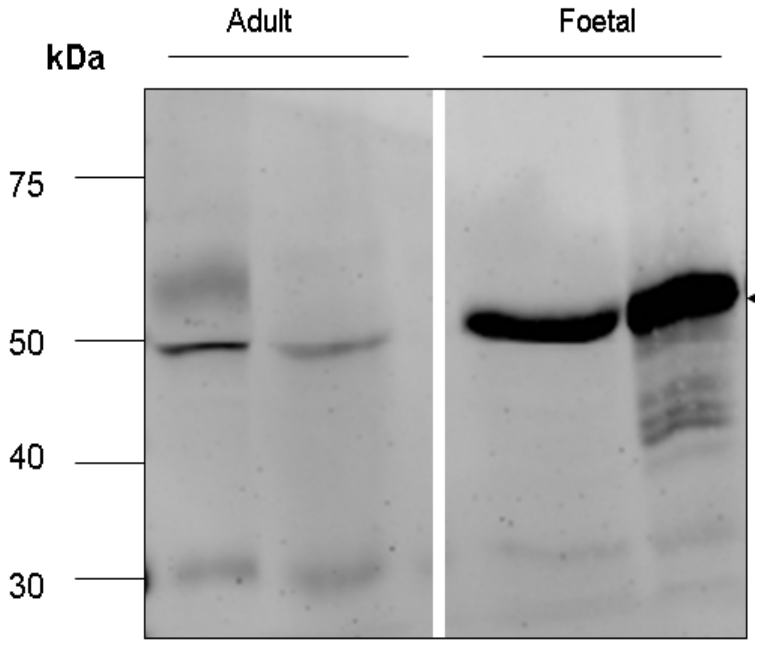


Figure 5.3. CELF proteins expression in adult and foetal rat brain.
Western blot analysis of adult and foetal rat brain homogenates were probed with the 3B1 antibody. CELF proteins are detected at 50 kDa in adult and foetal rat brain. Molecular weight markers are indicated on the left.

5.3 CELF proteins expression in human brain

To confirm the expression of CELF proteins in normal human brain, after their detection in rat brain, we performed a western blot analysis of homogenised normal human brain with 3B1 antibody. The monoclonal antibody, 3B1 binds to an epitope at the third RRM (RNA recognition motif) domain of all CELF proteins, detecting members of the CELF family of proteins such as CELF1, CELF3 and CELF4) (Good et al., 2000).

Total brain homogenates from three normal brain temporal cortices analysed by western blotting were probed with 3B1 antibody. Although the 3B1 antibody detects all members of the CELF family of proteins; we found that, independent of age, sex and postmortem delay, a single band of CELF proteins was present in the temporal cortex of all the normal human brain analysed (Figure 5.4). CELF proteins were detected at approximately 50 kDa when equal amounts of proteins are loaded, seen from the level of GAPDH (Figure 5.4). We therefore confirm that the 3B1 antibody recognises CELF proteins in normal human brain.

In subsequent experiments the 3B1 antibody will be used to compare the expression levels of CELF proteins in AD cases compared to age-matched control human brain.

5.3.1 Detection of CELF transcripts by RT-PCR

After verification of CELF proteins expression in cell lines, rat brain and normal human brain at the protein level using the 3B1 antibody, we wanted to investigate the expression of individual CELF proteins at the RNA level by RT-PCR. This was done because the 3B1 antibody detects all members of the CELF family of proteins. To determine the expression of individual CELF proteins at RNA level by RT-PCR, primers that detect each human CELF protein transcript including CELF1, CELF3 and CELF4 were designed. CELF 5 which is expressed predominantly in the brain was not studied because very little is known about it and its influence on tau exon 10 splicing is unknown at the moment.

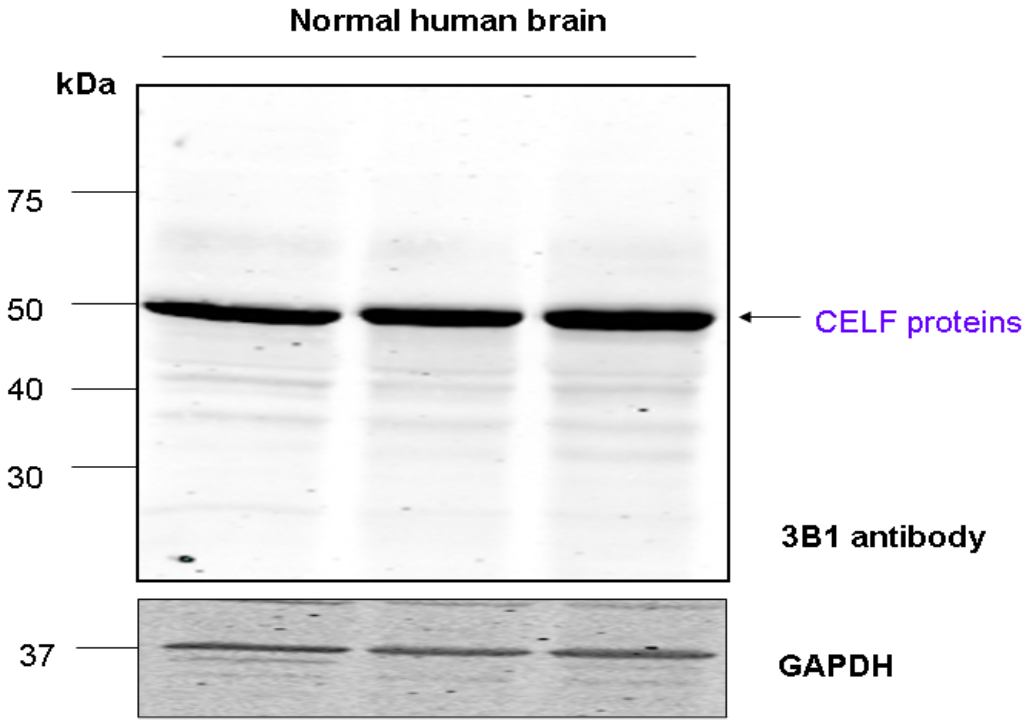


Figure 5.4. CELF proteins expression in normal human brain.

Total brain homogenates from three normal brain temporal cortices were analysed by western blotting and probed with the 3B1 antibody. CELF proteins were detected in normal human brain at 50kDa. GAPDH (37kDa) was used as a loading control. Molecular weight markers are indicated on the left.

5.3.1.1 Detection of CELF transcripts in transfected cells by RT-PCR

Total RNA was extracted from HeLa cells co-transfected with CELF1, CELF3 and CELF4 cDNA.

With CELF1 primers, CELF1 amplicon with a size of 236bp was detected in HeLa cells transfected with CELF1 cDNA (Figure 5.5A). Likewise, in HeLa cells transfected with CELF3 cDNA, a 209bp CELF3 amplicon was detected (Figure 5.5B). With CELF4 cDNA, a 236bp amplicon was detected with CELF4 primers after RT-PCR (Figure 5.5C).

In non-transfected HeLa cells, no amplicon was detected as expected because a higher number of PCR cycles are required to amplify endogenous CELF transcripts. GAPDH primers were used to detect endogenous GAPDH with a PCR product size of 496bp (Figure 5.5D). We show that the designed CELF primers are specific, only detecting their corresponding CELF transcripts in the transfected HeLa cells (Figure 5.5).

5.3.1.2 Detection of CELF1 transcripts in SH-SY5Y cells

To confirm endogenous expression of CELF1 transcripts in SH-SY5Y cells, total RNA extracted from these cells was reverse transcribed and PCR products amplified to detect CELF1 transcripts. CELF1 amplicons with a size of 236bp are detected in SH-SY5Y cells after RT-PCR analysis (Figure 5.6). This confirms endogenous CELF1 expression in SH-SY5Y cells.

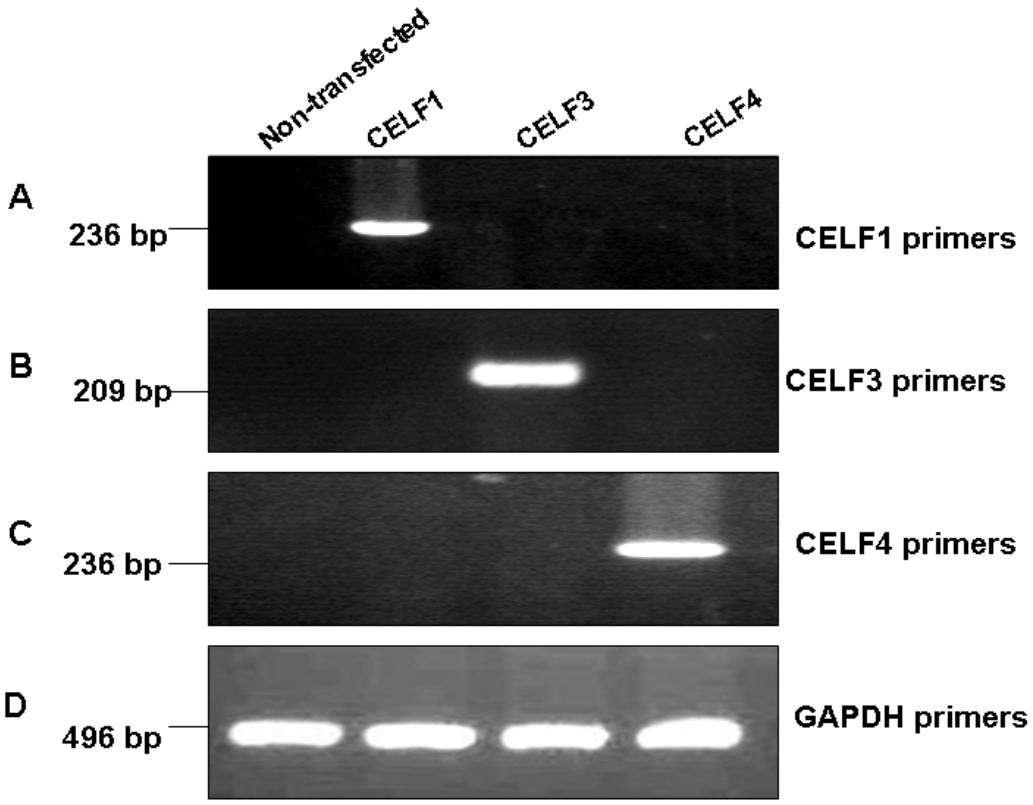


Figure 5.5. Detection of CELF protein transcripts in transfected cells.

HeLa cells were transfected with CELF1, CELF3 and CELF4 cDNA for 24 h. RNA extracted from non-transfected and transfected HeLa cells was reverse transcribed and PCR products were amplified using their corresponding primers (A-D).

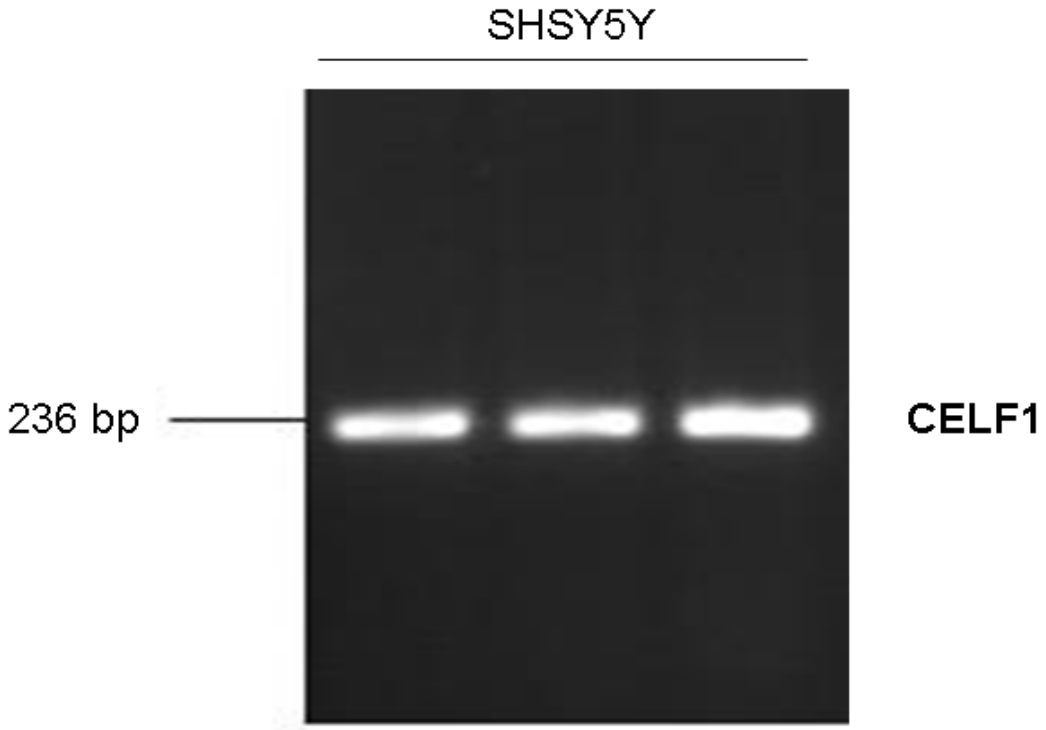


Figure 5.6. Detection of CELF1 in SH-SY5Y cells.

CELF1 is present in SH-SY5Y cells shown by the distinct amplicons in the three lanes at 236bp.

5.3.1.3 CELF protein transcripts expression by RT-PCR in human brain.

Total RNA extracted from three normal brain temporal cortices was reverse transcribed and amplified using specific CELF protein primers as detailed in the Methods chapter. The expression of CELF proteins including CELF1, CELF3 and CELF4 was assayed by RT-PCR and PCR products visualised using ethidium bromide (Figure 5.7).

RT-PCR analysis showed that CELF protein members; CELF1, CELF3 and CELF4 are expressed in the temporal cortices of normal human brain (Figure 5.7). These specific CELF protein primers will be used to determine the expression levels of relevant CELF proteins in brain regions affected in AD by RT-qPCR in subsequent sections.

5.4 CELF proteins expression in the regions affected in AD brain

Here, we investigate the expression levels of CELF proteins including CELF1, CELF3 and CELF4 in the amygdala and hippocampus of AD patients as well as other brain regions affected in AD including the frontal cortex and temporal cortex characteristically known to have a high ratio of neurofibrillary tangles in AD patient's brain (Braak and Braak, 1992). CELF5 was not included in this study because very little is kown about its function in relation to tau splicing and splicing in general. The cerebellum was used as a control brain region because it is largely unaffected in AD. Total RNA extracted from the frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum of fifteen AD cases without TDP-43 inclusions (ADTDP43-), eight AD cases with TDP43- inclusions (ADTDP43+) and eight age-matched normal control subjects was analysed by RT-qPCR described in the Methods chapter.

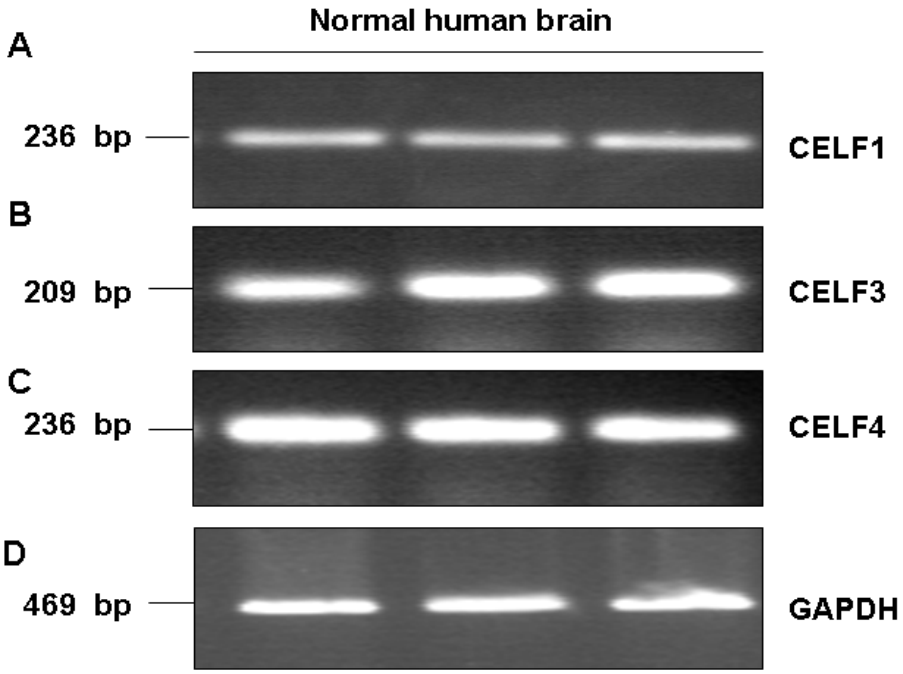


Figure 5.7. Expression of CELF transcripts in normal human brain.

The expression of CELF proteins (CELF1, CELF3 and CELF4) was assayed by RT-PCR using primers specific for each human SR protein transcript; GAPDH was used as a loading control. PCR products were visualised using ethidium bromide staining. Amplicon sizes of 236 bp, 209 bp, 236 bp and 469 bp were detected for CELF1, CELF3 and CELF4 and GAPDH respectively.

For all subsequent experiments, we considered the following criteria:

a) Control samples analysed were void of any pathology seen in AD brain such as tau aggregates or amyloid plaques.

b) GAPDH and β–actin house-keeping genes were used as reference genes to normalise RNA expression of CELF proteins; only cases with similar expression of GAPDH and β–actin were selected for analysis because of variability between samples such as post mortem delay and RNA quality. Data are normalized means with error bars indicating ± SEM of two independent experiments.

Total brain homogenates from the frontal cortex, temporal cortex, amygdala, hippocampus and cerebellum of three ADTDP43-, ADTDP43+ and age-matched normal control subjects were analysed by western blotting and probed with the 3B1 antibody. Throughout these experiments, the house-keeping gene used as a loading control was β-actin detected at 42 kDa. 3B1 antibody was used to determine the relative level of expression of CELF proteins at protein level. CELF protein expression is determined as a ratio of CELF protein and β-actin (CELF/β-actin).

5.4.1 CELF protein expression in the amygdala of AD brain

In Chapter three, we showed by RT-PCR that there was an increased expression of tau containing exon 10 (4R) in the amygdala of ADTDP43- compared to age-matched controls (Figure 3.3). By RT-qPCR, we found an increased expression of total tau levels only in the amygdala of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.6).

Here, we investigate by RT-qPCR the expression levels of CELF1, CELF3 and CELF4 RNA with the same primers that detected their expression in normal brains (Figure 5.7) in the amygdala.

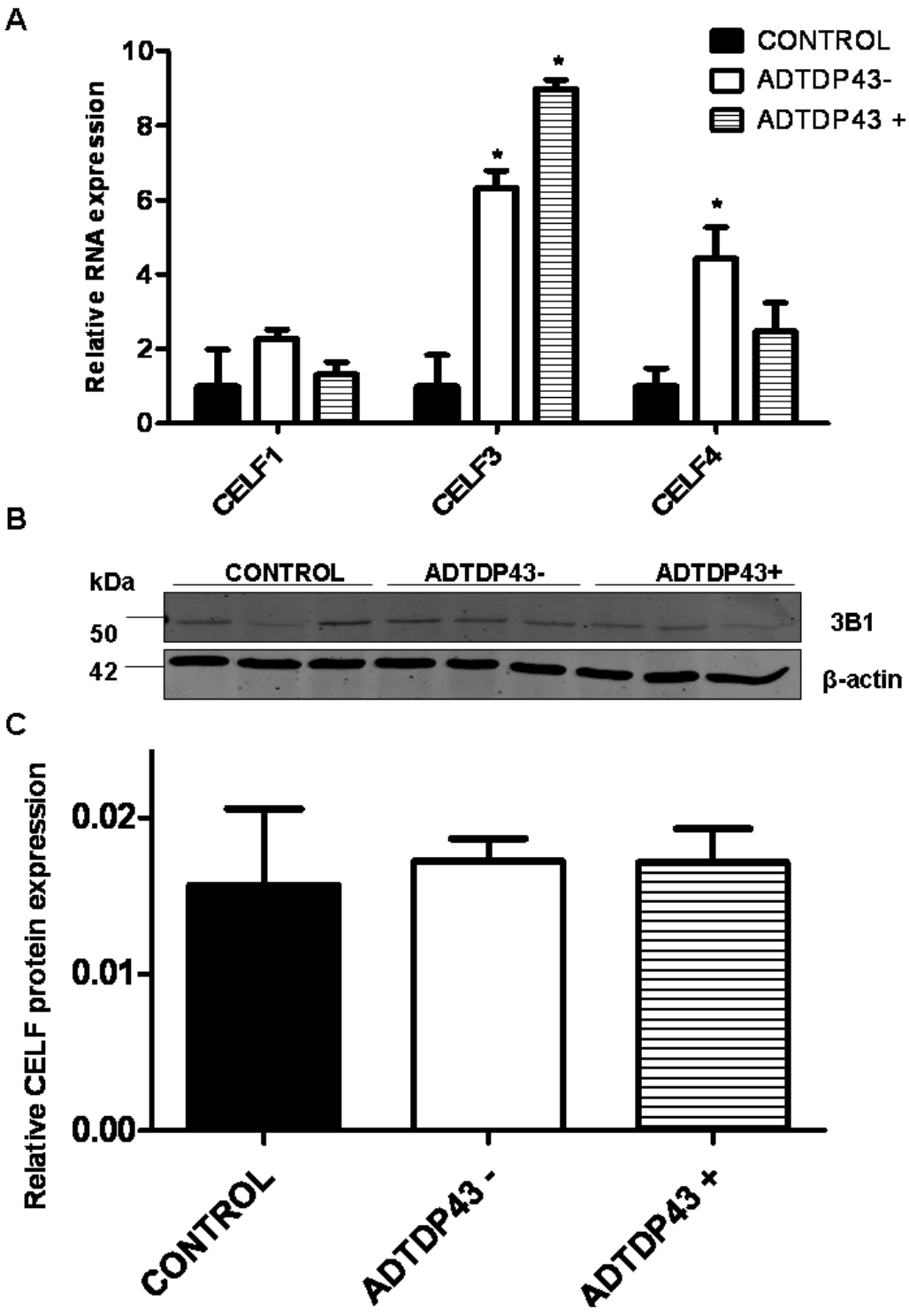
Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the amygdala. These include: control (C) cases, C1 and

C4 (RIN nos < 5); ADTDP43- (A) cases A2, A8, A11, A12, A13 and A15 (RIN nos < 5); A6 and A9 (Ct value > 28) while A12 and A14 (no tissue available); ADTDP43+ (T) cases T3, T4, T6 (RIN nos < 5) while T5 and T9 (Ct value > 28).

We found that the expression levels of CELF1 RNA did not change in the amygdala of ADTDP43- and ADTDP43+ cases compared to controls (Figure 5.8A). There was a significant 3.4-fold increase in CELF4 RNA expression in ADTDP43- cases compared to age-matched controls (4.43 ± 1.19 vs. 1.00 ± 0.83, $P < 0.05$). However, in ADTDP43+ cases, the expression of CELF4 RNA was not significantly different to that observed in controls (2.47 ± 0.76 vs. 1.00 ± 0.83) (Figure 5.8A). Interestingly, we found a 5.3-fold and 8-fold increase in the expression CELF3 RNA in ADTDP43- (6.32 ± 0.65 vs. 1.00 ± 1.18, $P < 0.05$) and ADTDP43+ (8.98 ± 0.24 vs. 1.00 ± 1.18, $P < 0.05$) cases respectively compared to age-matched controls (Figure 5.8A). In ADTDP43+ cases, the increase in CELF3 RNA was more prominent than that observed in ADTDP43- cases compared to age-matched controls. However, this increase was not significantly different when ADTDP43- and ADTDP43+ cases are compared (Figure 5.8A).

To determine whether these changes in CELF3 and CELF4 RNA are translated at protein level, we analysed brain homogenates from amygdala of three ADTDP43-, ADTDP43+ and age-matched controls by western blotting with the 3B1 antibody. We found the expression of CELF proteins at 50 kDa in all groups analysed when equal amounts of proteins were loaded seen from the levels of β-actin (Figure 5.8B). Measurement of the intensity of the CELF protein bands at 50 kDa relative to the amount of protein loaded (β-actin) was not different in ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.8C).

Figure 5.8. CELF proteins expression in the amygdala of AD human brain.
(A) CELF proteins expression in the amygdala of AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions and age-matched controls were analysed. CELF protein (CELF1, CELF3 and CELF4) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of CELF1 RNA in ADTDP43- and ADTDP43+ cases compared to controls. However, CELF4 RNA expression was significantly increased only in ADTDP43- cases compared to age-matched controls. There was a significant increase in CELF3 RNA expression in ADTDP43- and ADTDP43+ cases compared to age-matched controls. One-way ANOVA *, $P<0.05$; (Tukey's test). n, control = 6, ADTDP43- = 5, ADTDP43+ = 3. (B) Total brain homogenates from the amygdala of AD (ADTDP43- and ADTDP43+ cases) brain and controls were analysed by western blotting and probed with the 3B1 antibody. (C) Optical density measurements of the western blots showed that CELF proteins are expressed at similar levels in the amygdala of ADTDP43-, ADTDP43+ cases as well as controls with no significant differences. Significance was set at $P<0.05$, One way ANOVA.


A
CONTROL
ADTDP43-
ADTDP43 +
Relative RNA expression
10
8
6
4
2
0
*
*
*
CELF1
CELF3
CELF4
B
kDa
CONTROL
ADTDP43-
ADTDP43+
50
42
3B1
β-actin
C
Relative CELF protein expression
0.02
0.01
0.00
CONTROL
ADTDP43 -
ADTDP43 +

5.4.2 CELF protein expression in the frontal cortex of AD brain

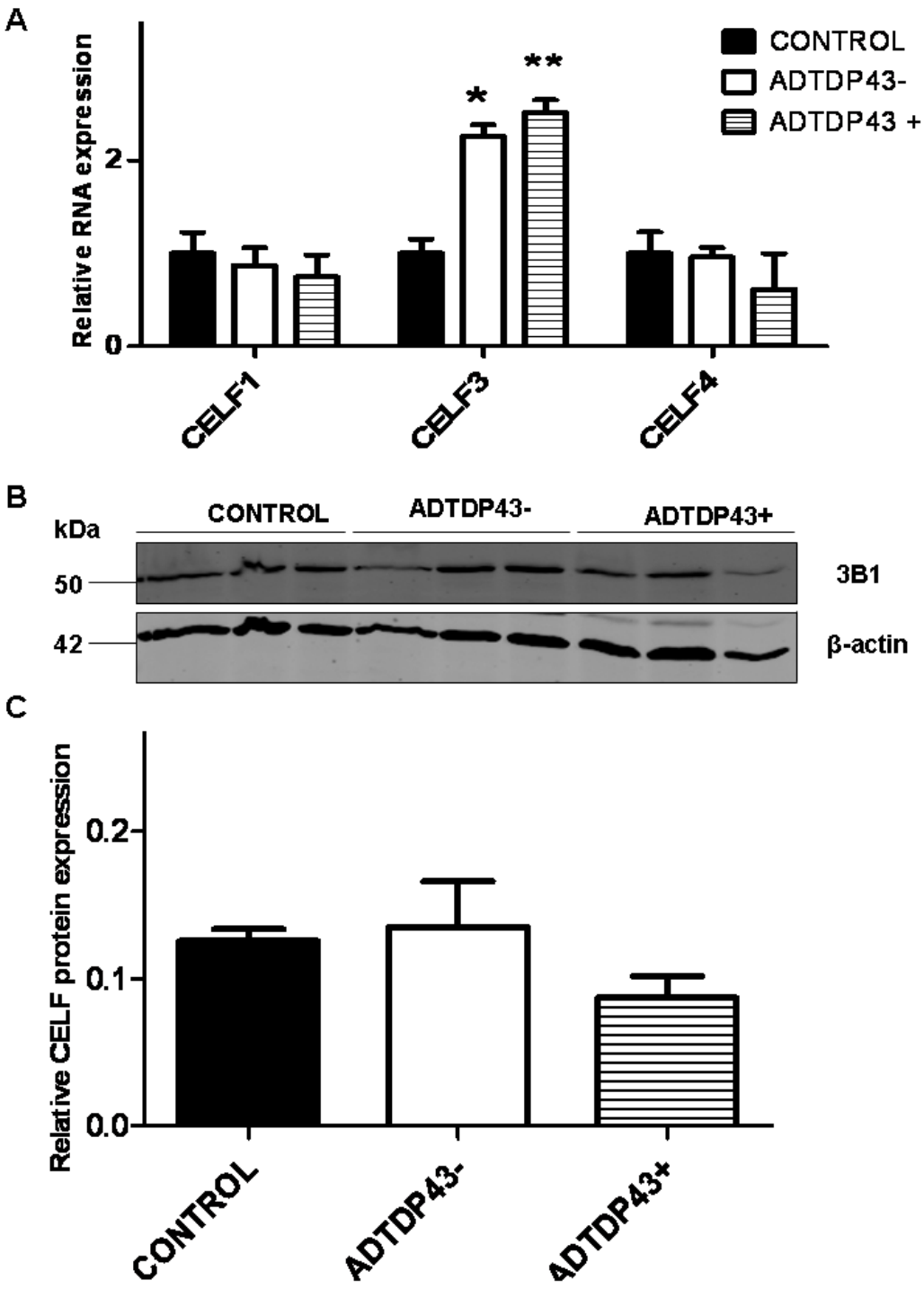
In Chapter three, we investigated the alternative splicing pattern of tau containing exon 10 in the frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls by RT-PCR. We did not find any significant difference in the percentage of 4R tau in frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.1). Similarly, there was no significant difference in total tau RNA expression in the frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.6).

Here, we investigated the expression levels of CELF1, CELF3 and CELF4 RNA in the frontal cortex by RT-qPCR. We found that the expression levels of CELF1 RNA did not show any significant difference in the frontal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.9A). Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the frontal cortex. These include: control (C) cases, C1 and C7 (RIN nos < 5); ADTDP43- (A) cases A7 and A12 (RIN nos < 5); A2, A6, A8, A10, A11, A13, A14 and A15 (Ct value > 28); ADTDP43+ (T) cases T3 and T4 (RIN nos < 5) while T5, T6 and T8 (Ct value > 28).

There was a significant 1.3-fold increase in CELF3 RNA expression in ADTDP43- cases compared to age-matched controls (2.26 ± 0.18 vs. 1.00 ± 0.22, $P < 0.05$) (Figure 5.9A). Interestingly, we also found a 1.5-fold increase in the expression of CELF3 RNA in ADTDP43+ (2.52 ± 0.19 vs. 1.00 ± 0.22, $P < 0.01$) cases compared to age-matched controls (Figure 5.9A). We investigated whether the increase in CELF3 RNA expression is translated at protein level in the frontal cortex. Brain homogenates from the frontal cortex of three ADTDP43-, ADTDP43+ cases and age-matched controls were analysed by western blotting and probed with 3B1 antibody. We show expression of varying amounts of CELF proteins at 50 kDa in all three groups analysed when equal amounts of proteins was loaded seen from the levels of β-actin (Figure 5.9B). However, there was no significant difference in the expression of CELF proteins at protein level in ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.9C).

Figure 5.9. CELF proteins expression in the frontal cortex of control and Alzheimer's disease (AD) brain.

(A) CELF proteins expression in the frontal cortex of AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions and age-matched controls were analysed. CELF protein (CELF1, CELF3 and CELF4) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of CELF1 and CELF4 RNA in ADTDP43- and ADTDP43+ cases compared to controls. However, there was a significant increase in CELF3 RNA expression in ADTDP43- and ADTDP43+ cases compared to control subjects. One-way ANOVA; *, $P<0.05$; **, $P<0.01$; (Tukey's test). n, control = 6, ADTDP43- = 5, ADTDP43+ = 3. (B) Total brain homogenates from the frontal cortices of ADTDP43- and ADTDP43+ cases and controls were analysed by western blotting and probed with 3B1 antibody. (C) Optical density measurements of the western blots showed that CELF proteins are expressed at similar levels in the frontal cortex of ADTDP43-, ADTDP43+ cases as well as controls with no significant differences. Significance was set at $P<0.05$, One way ANOVA.


A
Relative RNA expression
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2
CONTROL
ADTDP43-
ADTDP43 +
*
**
CELF1
CELF3
CELF4
B
kDa
CONTROL
ADTDP43-
ADTDP43+
50
42
3B1
β-actin
C
Relative CELF protein expression
0.0
0.1
0.2
CONTROL
ADTDP43-
ADTDP43+

5.4.3 CELF protein expression in the temporal cortex of AD brain

In the Chapter three, we investigated the alternative splicing pattern of tau containing exon 10 in the temporal cortex of ADTDP43- and ADTDP43+ cases compared to age-matched controls by RT-PCR. We found that there was an increased expression of 4R tau in the temporal cortex of ADTDP43- compared to age-matched controls but not in ADTDP43+ cases (Figure 3.2). However, the total tau level in the temporal cortex of ADTDP43- and ADTDP43+ cases was not significantly different when compared to age-matched controls (Figure 3.6).

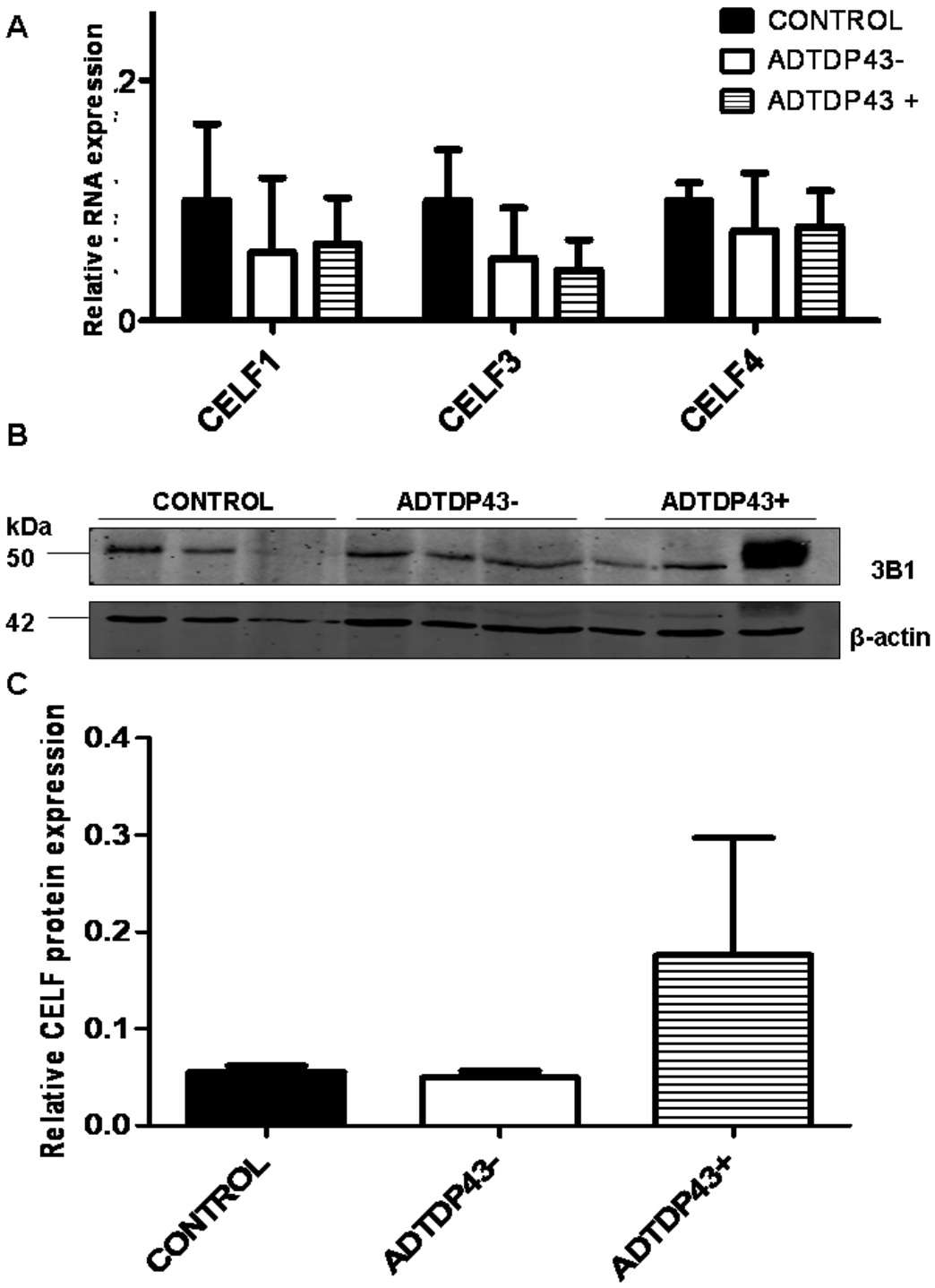
Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the temporal cortex; these include: Control (C) cases, C1 and C7 (RIN nos < 5); ADTDP43- (A) cases A7 and A13 (RIN nos < 5); A2, A4, A6, A9, A12, A14 and A15 (Ct value > 28); ADTDP43+ (T) cases T3, T4 and T5 (RIN nos < 5) while T9 (Ct value > 28).

Analysis of the temporal cortex by RT-qPCR showed that there was no significant difference in the expression levels of CELF1, CELF3 and CELF4 RNA in ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.10A).

Western blot analysis of brain homogenates from the temporal cortex of ADTDP43-, ADTDP43+ and age-matched controls with the 3B1 antibody showed varying amounts of CELF proteins expression at 50 kDa in all three groups analysed when equal amounts of proteins was loaded seen from the levels of β-actin (Figure 5.10B). In the controls subjects, CELF protein expression was variable although there were relatively equal amounts of protein in each lane. We observed a consistent expression of CELF proteins in ADTDP43- cases compared to controls (Figure 5.10B). Interestingly, one of the ADTDP43+ cases showed a prominent increase in CELF protein expression (Figure 5.10B). However, statistical analysis did not show any difference in CELF proteins expression at protein level in ADTDP43- cases compared to age-matched controls (Figure 5.10C). The increase in CELF protein expression observed in ADTDP43+ cases compared to age-matched controls was not significantly different (Figure 5.10C).

Figure 5.10. CELF proteins expression in the temporal cortex of control and Alzheimer's disease (AD) brain.

(A) CELF proteins expression in the temporal cortex of AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions and age-matched controls were analysed. CELF protein (CELF1, CELF3 and CELF4) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of CELF1, CELF3 and CELF4 RNA in the temporal cortex of ADTDP43- and ADTDP43+ cases compared to control. Significance was set at $P<0.05$ One way ANOVA. n, control = 6, ADTDP43- = 5, ADTDP43+ = 4. (B) Total brain homogenates from three temporal cortices of ADTDP43- and ADTDP43+ cases and controls were analysed by western blotting and probed with 3B1 antibody. A single band of CELF proteins was detected at 50 kDa with the 3B1 antibody. (C) Optical density measurements of the western blots showed that CELF proteins are expressed at similar levels in the temporal cortex of ADTDP43-, ADTDP43+ cases as well as controls with no significant differences. Significance was set at $P<0.05$, One way ANOVA.


A
CONTROL
ADTDP43-
ADTDP43 +
Relative RNA expression
2
0
CELF1
CELF3
CELF4
B
CONTROL
ADTDP43-
ADTDP43+
kDa
50
42
3B1
β-actin
C
Relative CELF protein expression
0.4
0.3
0.2
0.1
0.0
CONTROL
ADTDP43-
ADTDP43+

5.4.4 CELF protein expression in the hippocampus of AD brain

In Chapter three, we investigated the alternative splicing pattern of tau containing exon 10 (4R) in the hippocampus of ADTDP43- and ADTDP43+ cases compared to age-matched controls by RT-PCR. We found that there was an increased expression of 4R tau in the hippocampus of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 3.4). Conversely, the total tau level in the hippocampus of ADTDP43- and ADTDP43+ cases did not show any significant difference when compared to age-matched controls (Figure 3.6).

We investigated the expression levels of CELF1, CELF3 and CELF4 RNA in the hippocampus by RT-qPCR. Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the hippocampus; these include: Control (C) cases, C1 and C7 (RIN nos < 5); ADTDP43- (A) cases A1, A2, A4, A7, A10, A11 and A14 (RIN nos < 5); ADTDP43+ (T) cases T3, T4, T6 and T8 (RIN nos < 5); T2 (Ct value > 28).
RT-qPCR revealed that the expression levels of CELF1 RNA was not different in the hippocampus of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.11A). Similarly, there was no significant difference in the expression levels of CELF3 and CELF4 RNA in ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.11A).

Western blot analysis of brain homogenates from the hippocampus of ADTDP43-, ADTDP43+ and age-matched controls with 3B1 antibody showed varying expression of CELF proteins at 50 kDa in all three groups analysed (Figure 5.11B). Although there were relatively equal amounts of protein in each lane, we observed variability in the expression of CELF proteins (Figure 5.11B). Statistical analysis did not show any difference in the expression of CELF proteins at protein level in ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.11C).

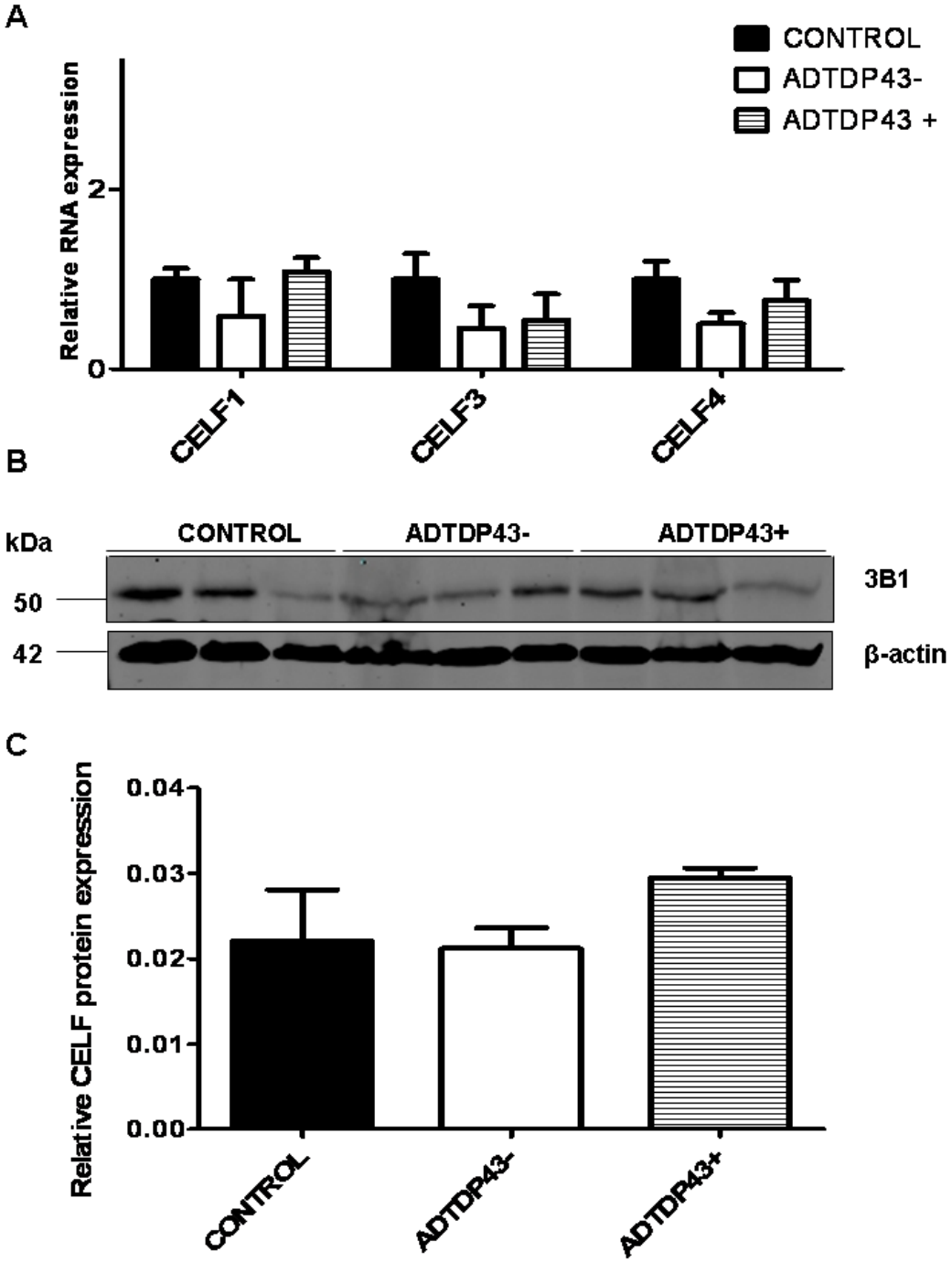

A
CONTROL
ADTDP43-
ADTDP43 +
Relative RNA expression
2
0
CELF1
CELF3
CELF4
B
kDa
CONTROL
ADTDP43-
ADTDP43+
50
42
3B1
β-actin
C
Relative CELF protein expression
0.04
0.03
0.02
0.01
0.00
CONTROL
ADTDP43-
ADTDP43+

Figure 5.11. CELF proteins expression in the hippocampus of control and Alzheimer's disease (AD) brain.

(A) CELF proteins expression in the hippocampus of AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions and age-matched controls were analysed. CELF protein (CELF1, CELF3 and CELF4) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of CELF1, CELF3 and CELF4 RNA in the hippocampus of ADTDP43- and ADTDP43+ cases compared to control. Significance was set at $P<0.05$ One way ANOVA. n, control = 6, ADTDP43- = 5, ADTDP43+ = 3. (B) Total brain homogenates from the hippocampus of ADTDP43- and ADTDP43+ cases and controls were analysed by western blotting and probed with 3B1 antibody. A single band of CELF proteins was detected at 50 kDa with the 3B1 antibody. (C) Optical density measurements of the western blots showed that CELF proteins are expressed at similar levels in the hippocampus of ADTDP43-, ADTDP43+ cases as well as controls with no significant differences. Significance was set at $P<0.05$, One way ANOVA.

5.4.5 CELF protein expression in the cerebellum of AD brain

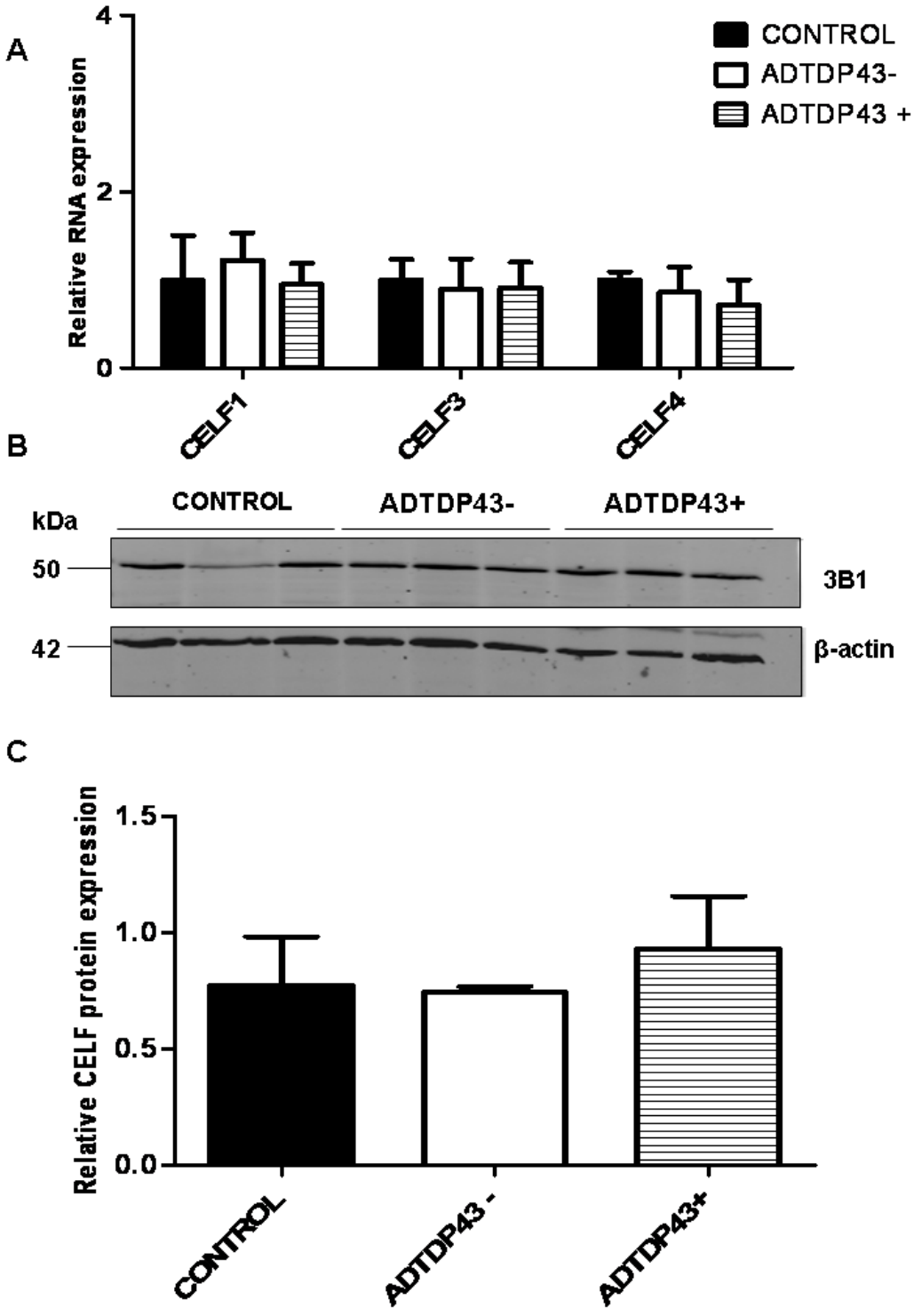
We examined the expression levels of CELF1, CELF3 and CELF4 RNA in the cerebellum by RT-qPCR. Some ADTDP43- and ADTDP43+ cases as well as control cases were excluded from the data analysis in the cerebellum. These include: control (C) cases, C1, C6 and C7 (RIN nos < 5); ADTDP43- (A) cases A8 (RIN nos < 5); A1, A4, A7, A9, A12 and A13 (Ct value > 28); ADTDP43+ (T) cases T3 (RIN nos < 5), T4, T5, T7 and T9 (Ct value > 28).
We found that the expression levels of CELF1, CELF3 and CELF4 RNA did not show any significant difference in the cerebellum of ADTDP43- and ADTDP43+ cases compared to age-matched controls (Figure 5.12A).

Western blot analysis of brain homogenates from the cerebellum of ADTDP43-, ADTDP43+ and age-matched controls with 3B1 antibody showed consistent expression of CELF proteins at 50 kDa in the cerebellum of ADTDP43-, ADTDP43+ and age-matched controls when equal amounts of proteins were loaded seen from the levels of β-actin (Figure 5.12B). In the three controls with equal amounts of protein loaded, CELF proteins expression was variable in one of the control subjects (Figure 5.12B). Statistical analysis of CELF protein expression was not different in ADTDP43-, ADTDP43+ and age-matched controls (Figure 5.12C).

A summary of the RT-qPCR analysis for CELF1, CELF3 and CELF4 is shown in Figure 5.13. RT-qPCR showed that the expression level of **CELF1 RNA** was not different in all five brain regions of ADTDP43- and ADTDP43+ cases compared to age-matched controls. However, **CELF4 RNA** expression was significantly increased only in the amygdala of ADTDP43- cases compared to controls. There was no significant difference in the expression of CELF4 RNA in ADTDP43- and ADTDP43+ cases compared to controls in the frontal cortex, temporal cortex, hippocampus and cerebellum. **CELF3 RNA** expression was significantly increased in the frontal cortex and amygdala of ADTDP43- and ADTDP43+ cases compared to controls. There was no significant difference in the expression of CELF3 RNA in the temporal cortex, hippocampus and cerebellum of ADTDP43- and ADTDP43+ cases compared to controls.

Figure 5.12. CELF proteins expression in the cerebellum of control and Alzheimer's disease (AD) brain.

(A) CELF proteins expression in the cerebellum of AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions and age-matched controls were analysed. CELF protein (CELF1, CELF3 and CELF4) expression was assayed by RT-qPCR and normalised to β-actin and GAPDH. RT-qPCR showed that there was no significant difference in the expression of CELF1, CELF3 and CELF4 RNA in the cerebellum of ADTDP43- and ADTDP43+ cases compared to control. Significance was set at $P<0.05$ One way ANOVA. n, control = 6, ADTDP43- = 5, ADTDP43+ = 3. (B) Total brain homogenates from the cerebellum of ADTDP43- and ADTDP43+ cases and controls were analysed by western blotting and probed with 3B1 antibody. A single band of CELF proteins was detected at 50 kDa with 3B1 antibody (C) Optical density measurements of the western blots showed that CELF proteins are expressed at similar levels in the cerebellum of ADTDP43-, ADTDP43+ cases as well as controls with no significant differences. Significance was set at $P<0.05$, One way ANOVA.


A
CONTROL
ADTDP43-
ADTDP43 +
Relative RNA expression
4
2
0
CELF1
CELF3
CELF4
B
CONTROL
ADTDP43-
ADTDP43+
kDa
50
42
3B1
β-actin
C
Relative CELF protein expression
1.5
1.0
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0.0
CONTROL
ADTDP43 -
ADTDP43+

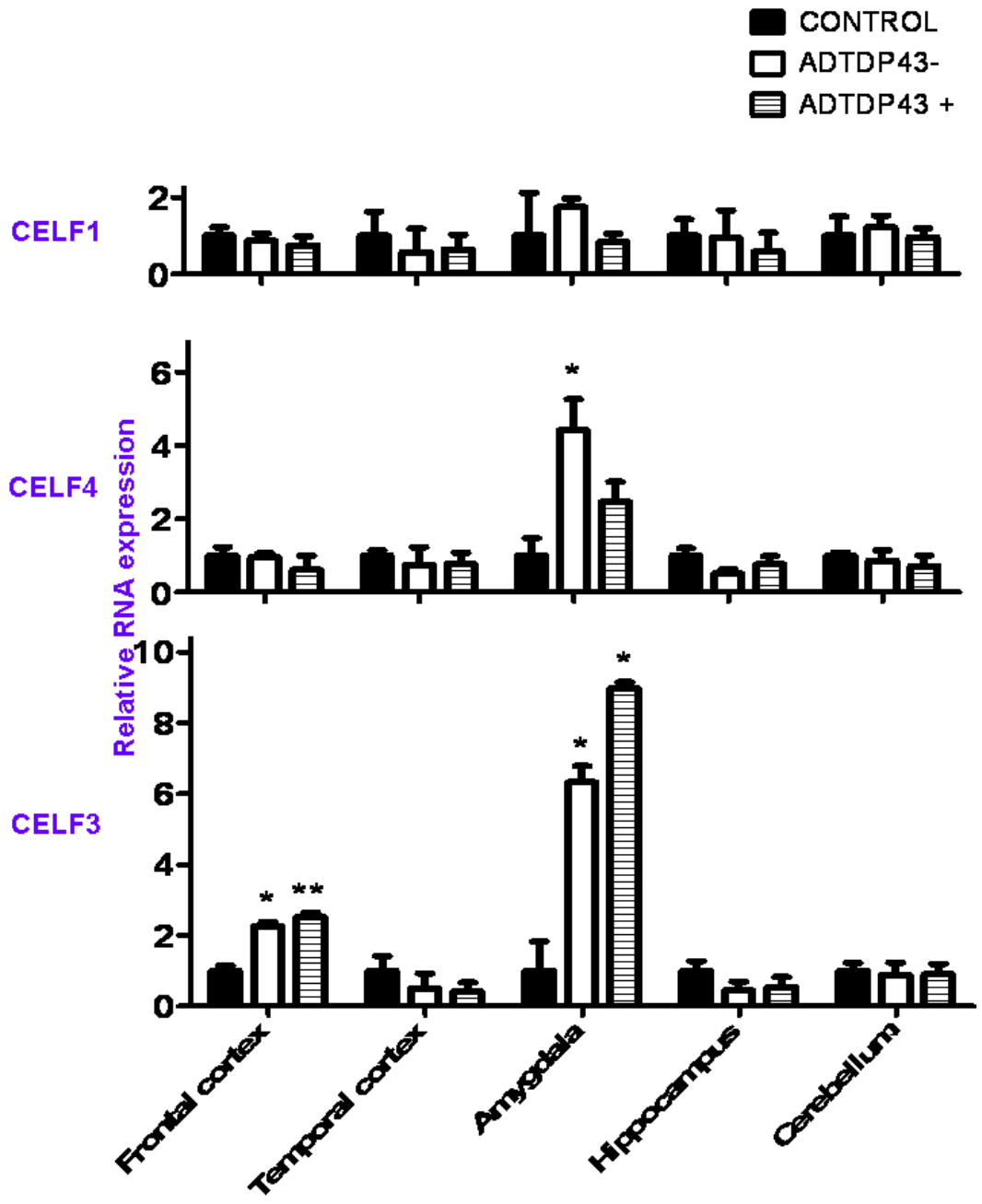

Figure 5.13. CELF proteins expression in brain regions of AD brain.

CELF proteins expression in AD brain with (ADTDP43+) and without (ADTDP43-) TDP-43 inclusions of four brain regions affecte d in AD (frontal cortex, temporal cortex, amygdala and hippocampus) and one region largely unaffected in AD, the cerebellum; was assayed by quantitative RT-PCR and normalised to GAPDH and β-actin. Data are normalised mean values with error bars indicating ± SEM. One-way ANOVA *, $P<0.05$; **, $P<0.01$; (Tukey's test)

5.5 Summary

The results presented in this chapter demonstrate that:

- CELF protein transcripts regulating tau exon 10 splicing including; CELF1, CELF3 and CELF4 are expressed at the RNA level in normal human brain.

- One band at 50 kDa corresponding to CELF1 or other members of the CELF family of proteins is expressed at the protein level in normal human brain detected with 3B1 antibody.

- CELF4 is increased in the amygdala of AD cases without TDP-43 inclusions (ADTDP43-).

- There is an increased expression of CELF3 in the amygdala and frontal cortex of AD cases irrespective of the presence of TDP-43 inclusions compared to controls which suggest that in the presence of TDP-43 inclusions, CELF3 RNA expression is increased.

Taken together, these findings show the expression profile of CELF proteins in normal human brain and as well as brain regions affected in AD (Figure 5.13). We show that the expression levels of CELF proteins (CELF3 and CELF4) that regulate tau splicing is altered in brain regions affected in AD. Taken together, our data confirms our hypothesis that the expression levels of splicing factors such as CELF3 and CELF4 may be altered, resulting in abnormal splicing activity in sporadic AD.

Chapter Six

6 Discussion

Mutations in the *MAPT* gene cause FTDP-17 due to abnormal tau exon 10 splicing (Hutton et al., 1998). Additionally, the presence of tau exon 10 missplicing in sporadic tauopathies such as PiD (3R tau overexpression), CBD and PSP (4R tau overexpresssion) suggests that splicing factors that regulate tau exon 10 splicing may contribute to the pathogenesis of these diseases. Furthermore, it indicates that neurodegeneration can be driven in humans regardless of which tau isoform is favoured. Elucidation of the underlying mechanisms by which these changes in tau alternative splicing affect tau's cellular function and result in neurodegeneration is a focus of scientific interest.

Understanding the exact function of RNA-binding proteins in tauopathies may be central to elucidating their role in the pathogenesis of sporadic tauopathies. RNA-binding proteins play an essential role in splicing and their aberrant expression and/or activity leads to several neurological disorders as seen in myotonic dystrophy (Ladd et al., 2001). Whether the abnormal expression and/or activity of splicing factors in tauopathies are decisive to tau splicing deficit remains elusive. The expression levels of CELF proteins and SR proteins involved in regulation of tau splicing has not been studied in sporadic tauopathies. This thesis has characterised the alternative splicing pattern of tau exon 10 as well as the expression profile of members of two families of RNA-binding proteins; SR proteins and CELF proteins in sporadic AD cases with and without TDP-43 inclusions (ADTDP43+ and ADTDP43-).

6.1 Aberrant tau splicing in AD human brain

Aberrant tau exon 10 splicing had been previously described in brain regions of sporadic tauopathies including CBD, PSP, PiD and AD (Chambers et al., 1999; Takanashi et al., 2002; Umeda et al., 2004; Yasojima et al., 1999); (Connell et al., 2005). We have now analysed the tau exon 10 splicing pattern in sporadic AD

cases with and without TDP-43 inclusions. We found a significant increase in 4R tau in vulnerable brain regions affected in AD. Specifically, in the amygdala of ADTDP43- cases where there is extensive NFT pathology, we found an increase in 4R/3R tau ratio by approximately 78.8 % compared to age-matched controls. Our findings are supported by increased 4R tau observed in the temporal cortex of sporadic AD cases previously determined by Glatz et al. (Glatz et al., 2006). In the frontal cortex, a region that is heavily affected with NFT pathology during the late stages of AD, we found that the percentage of 4R tau in AD cases irrespective of TDP-43 pathology was similar to that expressed in normal human adult brain. In addition, in the hippocampus, one of the brain regions first affected in AD, we found that 4R/3R tau ratio in AD cases without TDP-43 pathology was significantly increased by approximately 73 %.

This disturbance of the tau 4R/3R expression from the normal approximate ratio of 1:1 results in enhanced NFT formation and neurodegeneration (Andreadis, 2005). The pathological consequence of disturbed 4R/3R ratio where more 4R is favoured often alters tau function resulting in several pathogenic mechanisms. Hence, the equal expression of 4R tau and 3R tau in normal adult human brain is essential to maintain normal neuronal tau functions (Adams et al., 2010). These findings propose that altering the normal equimolar 4R/3R tau ratio may contribute to tau pathology in sporadic AD. The splicing pattern expression of the tau isoforms is regulated by RNA-binding proteins which are vital in determining the equimolar 4R/3R tau ratios.

The RT-PCR technique used in this study to determine the 4R/3R tau ratios does not indicate which cell types possess this increased expression of 4R tau. However, Goedert et al. showed that hippocampal pyramidal neurons and granule cells of the hippocampus, a region with early tau pathology, are vulnerable in AD brain (Goedert et al., 1989a; Goedert et al., 1989b). Interestingly, in the cerebellum, one of the brain regions spared in AD, we observed that 4R tau expression in ADTDP43- pathology was significantly increased. It is possible that the mechanism of tau missplicing is not specific to brain regions susceptible to NFT pathology only or that the changes observed in the cerebellum are very subtle and not sufficient to cause NFT pathology in the convoluted brain region.

The reason why NFT pathology develops in specific brain areas is not yet understood.

The intensities of the 4R tau and 3R tau isoform bands obtained within the AD groups (ADTDP43- and ADTDP43+ cases) varied greatly probably due to severity of symptoms and/or variation between individual AD patients as well as brain areas. This could be because AD cases with more severe NFT pathology show dramatic increase in the percentage 4R tau (Figures 3.2, 3.3, 3.4 and 3.5). The study of tau exon 10 splicing in sporadic AD has revealed conflicting results probably due to variability between studies and AD subjects used. For instance, some studies do not show any differences in exon 10 splicing in AD (Boutajangout et al., 2004; Chambers et al., 1999; Connell et al., 2005; Ingelsson et al., 2006). Although we did not determine whether the increase in 4R tau isoforms was translated to the protein level, we wanted to elucidate whether the expression of splicing factors regulating tau exon 10 splicing are altered at the RNA level in AD brain.

As predicted in the hypothesis, there was an increase in the expression of exon 10 in the amygdala and hippocampus, regions shown to possess extensive neuropathology (Appendix 1.1). We have now discerned using this RT-PCR approach that tau splicing is infact aberrant in these ADTDP43- cases. The possible explanation for this abnormal tau exon 10 splicing in brain regions (amygdala and hippocampus) affected by AD is that there is abnormal regulation of the splicing mechanism in brain regions with NFT pathology of AD patients. However, the change in transcript levels in the cerebellum in the absence of pathology in most AD cases casts doubt on the relevance of the approach to disease. Although the cerebellum is considered as a region without any pathology in AD patients yet, the reason for this an increase in the 4R/3R tau ratio in ADTDP43- cases is unknown.

Moreover, tangle development is unlikely to drive the shift in increased 4R tau expression in the amygdala and hippocampus because the cerebellum which is not normally associated with NFT pathology also showed an increased expression of 4R tau. The fact that mutations in the *MAPT* gene have not been associated with

AD (Poorkaj et al., 2001) suggests that other factors involved in the regulation of tau exon 10 splicing may disrupt tau splicing and contributes to the pathogenesis of AD. Hence, splicing factors may be genetic modifiers of disease pathology through their influence and control of tau exon 10 splicing (Nissim-Rafinia and Kerem, 2002). Our data suggest that abnormal splicing regulation of tau exon 10 splicing results in favourable expression of 4R tau which may subsequently promote tangle formation.

6.2 RNA-binding proteins in human brain.

The processes of RNA metabolism involves several proteins that mediate and regulate all aspects of RNA processing. RNA-binding proteins themselves are subject to post-transcriptional and post-translational modifications such as alternative splicing and phosphorylation respectively. They are also involved in RNA processing, mRNA transport, mRNA stability and mRNA translation (Anthony and Gallo, 2010). Tau exon 10 splicing is under the synergistic control of *cis*-acting elements and *trans*-acting factors. As seen in sporadic tauopathies, in the absence of mutations within the *MAPT* gene and predisposing haplotypes, tau pathology still develops. RNA-binding proteins involved in the regulation of tau exon 10 alternative splicing may contribute to pathogenesis of sporadic AD due to altered expression or activity. Furthermore, the expression profiling of RNA-binding proteins involved in tau alternative splicing in AD brain is important to elucidate a potential mechanism by which tau splicing is misregulated. This is because the expression level of splicing factors including SR proteins is closely related to their regulatory activity in specific adult brain tissues (Grabowski and Black, 2001) (Hsu et al., 2011). However, the activity of most splicing factors is not solely dependent on their abundance, but also regulated by their phosphorylation state (Nissim-Rafinia and Kerem, 2002). We characterised the expression of *trans*-acting RNA-binding proteins regulating tau mRNA processing.

6.2.1 Expression of SR proteins in human brain

SR proteins have previously been detected in cell lines and rat brain at the RNA and protein level (Daoud et al., 1999; Zahler et al., 1992). We have shown in normal human brain that SR proteins including SC35, SRp40 and SRp55 are expressed at RNA level as well as the presence of bona fide members of the SR protein family in normal human brain using mAb104. The mAb104 detects a phosphoepitope in the RS domain of all SR proteins (Zahler et al., 1992). This antibody was used to show expression of SR proteins in normal human brain.

We showed that the mAb104 antibody recognises SR proteins in different cell lines and rat brain similar to that seen in the literature (Delacourte et al., 1998; Gui et al., 1994b). The variation we observed in the expression of SR proteins at protein level in normal human brain could be due to posttranslational modifications such as phosphorylation (Hanamura et al., 1998). mAb104 also detects other bands in the three normal brain; these may represent other members of the SR protein family for example, 9G8 with a molecular weight size of 36 kDa (Huang and Steitz, 2001). Hence, we show the expression of SR proteins at the RNA and protein level in normal human brain before investigating by RT-qPCR, the RNA levels of specific SR proteins involved in the regulation of tau exon 10 splicing in brain regions of sporadic AD patients.

6.2.2 CELF proteins expression in human brain

All members of the CELF proteins family are expressed at RNA level in the human brain (Ladd et al., 2001; Ladd et al., 2004). Our findings confirm this by showing the expression of CELF1, CELF3 and CELF4 at RNA level in normal human brain. Some CELF proteins are involved in the regulation of tau splicing including CELF3 and CELF4 *in vitro* (Chapple et al., 2007; Dhaenens et al., 2011; Wang et al., 2004). The 3B1 antibody detects several members of the CELF family of proteins in cell lines, rat brain and human brain at the same size of 50 kDa. Hence, to determine specific members of the CELF family at protein level, in normal human brain, specific antibodies for each CELF protein will be required which are not readily available at present. The ability of CELF protein antibodies,

originally raised against one specific family member (CELF1), to identify multiple CELF proteins, is due to the sequence homology that exists between CELF proteins. Although there is not much similarity between CELF family members in the divergent domain, RRMs are well conserved across the protein family especially at RRM3 which is recognised by the 3B1 antibody.

The fact that only a single band is detected in normal human brain may be due to the relative expression of different CELF proteins. Consequently, it is difficult to ascertain which specific CELF proteins are expressed in the human brain. In humans, CELF4 is ubiquitously expressed but enriched in the hippocampus relative to the other members of the CELF family including the brain-specific CELF3 and CELF5 (Ladd et al., 2001; Ladd et al., 2004).

The expression of individual CELF proteins at the RNA as well as the detection of CELF proteins at the protein level in normal human brain allowed us to further investigate by RT-qPCR, the RNA levels of specific CELF proteins involved in the regulation of tau exon 10 splicing in brain regions of sporadic AD.

6.3 Up-regulation of RNA-binding proteins in AD brain

We used RT-qPCR to investigate the expression levels of members of the SR proteins and CELF proteins transcripts in AD brain. Comparisons between the expression levels of CELF proteins (CELF1, CELF3 and CELF4) and SR proteins (SC35, SRp40 and SRp55) in AD brain regions and controls were made.

The results obtained were independent of postmortem delay of the postmortem brain tissue showing no statistical correlation. The quantity and quality of RNA produced from each individual subject as well as the regions in AD and normal brain was very variable. However, most of the brain samples used had good RNA integrity number (RIN) number ($\geq$ 5) and we used more than one house keeping gene for data normalisation. After data analysis, three genes (CELF4, SRp40 and SRp55) were differentially expressed only in the amygdala of AD brain although one gene (CELF3) out of the six genes was differentially expressed in the amygdala and frontal cortex of AD irrespective of TDP-43 inclusions. Since the

amygdala is the most prominent region with differential expression, it is possible that there is a specific role for RNA-binding proteins in the amygdala that is not found in other brain regions.

6.3.1 Expression profile of some SR proteins in the amygdala of AD brain

Enhancer elements (such as SC35-like enhancer, PPE and ACE) present within tau exon 10 are recognised by SR proteins which promote tau exon inclusion (Fu, 1995; Stamm et al., 1994). Moreover, SR proteins modulate alternative splicing both *in vitro* and *in vivo* in a concentration dependent manner (Caceres et al., 1994; Fischer et al., 2004). Hence, SR proteins are an appealing target in the analysis of molecular changes that cause discrepancies in alternative splicing.

Earlier, we described marked changes in tau exon 10 alternative splicing in sporadic AD cases with and without TDP-43 inclusions. In the Chapter four we focussed our studies on the expression profiles of SR proteins splicing factors, including SC35, SRp40 and SRp55 in affected brain regions with known NFT pathology in sporadic AD cases. Evaluation of their expression pattern by RT-qPCR showed that in ADTDP43- brain, there were increased RNA levels of SRp40 and SRp55.

One of the aims of this study was to examine the expression pattern of different nuclear SR proteins in sporadic AD cases and to compare these with physiologically normal human brain. SR proteins are known to be potential regulators of several pre-mRNA processing including tau thereby influencing their cellular behaviour (Fischer et al., 2004). SC35 RNA, which promotes tau exon 10 inclusion through the SC35-like enhancer (Qian et al., 2011b) showed no significant difference in expression in all four brain regions affected in AD whereas a highly significant increase in the expression of SRp40 and SRp55 RNA was seen in the amygdala of ADTDP43- cases. SRp40 promotes tau exon 10 inclusion *in vitro* through its binding to an ESE within tau exon 10 (Kondo et al., 2004; Wang et al., 2005) while SRp55 inhibits tau exon 10 inclusion *in vitro*

through its activity on an ESS within tau exon 10 (Glatz et al., 2006; Kondo et al., 2004; Wang et al., 2005).

Consistent with this, we found an increase in 4R/3R tau ratio in the amygdala of sporadic ADTDP43- patients that also showed a significant increase in SRp40 RNA. However, we anticipated that if SR proteins that promote tau exon 10 inclusion (for instance SRp40) were increased in the amygdala of ADTDP43- brain, then SR proteins such as SRp55 which inhibits tau exon 10 inclusion would be decreased in the amygdala. However, we did not find this in our study as there is no clear correlation of the 4R/3R tau ratio with the increased expression of SR proteins in the amygdala or with any pathology. In addition, based on the fact that the changes seen in these brain regions show no correlation with the increase in 4R/3R tau ratios or any pathology, the changes seen may be global. It is not clear whether SRp55 inhibits tau exon 10 inclusion *in vivo* because data obtained from minigene studies must be interpreted cautiously since transcripts derived from minigenes may not act in the same manner as the full-length pre-mRNA. The increased tau exon 10 inclusion in the amygdala of ADTDP43- cases may be a consequence of the increased expression of SRp40 and SRp55 either individually or combined, by promoting the recognition of exon 10 as well as recruiting other splicing factors to tau pre-mRNA during splicing regulation. The fact that SC35 did not show a marked increase in the amygdala of ADTDP43- brain suggests that there are gene-specific alterations as well as brain region-specific alterations rather than a non-specific increase of the general RNA processing machinery. Interestingly, in ovarian cancer, the expression levels of SRp40 and SRp55 mRNA remained constant while SC35 mRNA was significantly increased (Fischer et al., 2004). Nevertheless, additional studies are required to clarify the comprehensive mechanism behind tau splicing regulation.

SR proteins play a vital role during spliceosome assembly and splicing, particularly in tau splicing. However, the aberrant increase in these SR proteins expressions was specific to the amygdala of ADTDP43- cases which suggests a special role for SR proteins within this brain region. It is possible that this is a secondary effect due to a general up-regulation of RNA-binding proteins when there is an abnormal environment or recruitment from nuclear speckles is initiated

but if this were the case, we would expect to see the same effect within other brain regions affected in AD. At this point, we can only speculate that changes in the abundance of SR proteins may cause abnormal splicing of other pre-mRNA transcripts modulated by these specific SR proteins. Since alternative splicing of individual pre-mRNA is regulated by a myriad of other splicing (often antagonistic) factors; we did not study the expression levels of other mRNAs regulated by SRp40 and SRp55 in the amygdala of our cohort AD cases. This combined control of splicing factors may explain why we found alterations in tau exon 10 splicing in the cerebellum since other splicing factors may modulate tau exon 10 inclusion in this brain area.

In addition to the analysis of tau exon 10 splicing in this study, these findings support the hypothesis that alterations in the expression of SR proteins splicing factors may cause changes in tau exon 10 splicing and contribute to the pathogenesis of AD. Furthermore, this may be crucial to the development of tau pathology in other sporadic tauopathies as well as AD. It would be essential to determine the expression levels of these SR proteins (SRp40 and SRp55) at protein level in the amygdala of AD cases to establish whether their expression is up-regulated due to amplification of their genes in ADTDP43- cases.

Although we did not find any significant difference in SR protein expression in the frontal cortex, temporal cortex and hippocampus of sporadic AD cases when compared to age-matched controls, our results provide evidence that SR protein expression is altered in ADTDP43- cases and may contribute to the altered tau exon 10 splicing observed in sporadic AD cases. Even though we found a significant increase in the expression of 4R tau in the cerebellum of AD cases irrespective of TDP-43 inclusions, we did not find a significant difference in the expression of any SR protein that promotes tau exon 10 inclusion.

Nissim-Rafinia et al. suggested that, during changes to the physiological conditions and/or the stage of cell development in normal tissues, differences in the levels of alternatively spliced transcripts arise due to modifications in the system regulating alternative splicing (Nissim-Rafinia and Kerem, 2002). Specific tissues show relative differences in the abundance of splicing factors *in vivo*.

These include SR proteins and hnRNP families which regulate tissue specific differences in splicing patterns (Hsu et al., 2011). Indeed, overexpression of splicing factors regulates the splicing pattern of alternatively spliced exons. Another level of regulation of splicing factors is their subcellular distribution which also controls splicing efficiency (Nissim-Rafinia and Kerem, 2002). It remains to be determined, whether the subcellular localisation of SR proteins are misregulated from nuclear speckles in the nucleus to the cytoplasm during aberrant cellular conditions in AD.

Altered tau exon 10 splicing causes a change in expression of tau isoforms (4R and 3R tau) in some FTDP-17 cases. Similarly, other disorders such as breast and colon cancers show changes in expression of some mRNA transcripts due to aberrant alternative splicing mechanism. The CD44 family are surface glycoproteins involved in cell migration and cell-matrix interactions (He et al., 2004). The *CD44* gene contains ten alternative exons, alternatively spliced into several CD44 isoforms (Nissim-Rafinia and Kerem, 2002). Alternative splicing of the CD44 pre-mRNA has been implicated as central in a number of human cancers, such as breast cancer (Stickeler et al., 1999). Other cancers that show high expression of alternatively spliced CD44 variants include colon and ovarian cancers while normal tissues only express standard version of CD44 (Fischer et al., 2004; He et al., 2004). The fact that changes in regulation of alternative splicing are due to altered SR proteins expression during breast cancer development was studied in a mouse model for mammary tumourigenesis. During tumourigenesis, there were prominent increases in the levels of CD44 transcripts as well as the expression levels of individual SR proteins (Stickeler et al., 1999). Only two SR proteins were prominently expressed in normal mature epithelial cells SRp75 and SRp55 while SRp40 and SRp30 showed a small increase during different stages of tumour development. In contrast, adenocarcinoma cells as well as lung and liver metastases, showed high expression levels of SR proteins suggesting that aberrant expression of SR proteins was induced during tumourigenesis (Stickeler et al., 1999). Furthermore, the multidrug resistance protein 1 (MRP1) is highly expressed in the ovarian tumour cells compared to matched normal tissues and correlates with increased expression of SRp20 (He et

al., 2004). Hence, alternations in splicing regulation play a role as a genetic modifier in disease.

6.3.2 Aberrant expression of CELF proteins in AD brain

Another aim of this study was to examine the expression patterns of different CELF proteins in sporadic AD cases and to compare these with physiologically normal human brain. CELF proteins are known to be potential regulators of several pre-mRNA processing including tau (Ladd et al., 2004). In DM1 patients, up-regulation of CELF1 and sequestration of MBNL1 are implicated as the major contributors to DM pathogenesis which also possess tau pathology in the brain. Hence, CELF proteins, which are modulators of tau alternative splicing, are interesting targets in the quest to determine the molecular changes that result in aberrant tau splicing in sporadic AD. Several CELF proteins that regulate tau exon 10 inclusion *in vitro* include CELF3 and CELF4 (Chapple et al., 2007; Wang et al., 2004). We evaluated their expression by RT-qPCR. We found that in the amygdala and frontal cortex of AD brain (regardless of TDP43 inclusions) there were increased RNA levels of CELF3 as well as CELF4 in the amygdala of ADTDP43- cases.

6.3.2.1. The expression of CELF4 proteins in the amygdala of AD brain is misregulated

The amygdala is primarily responsible for controlling emotions, learning and memory particularly reflective emotions such as fear and anxiety. The amygdala is one of the brain regions affected during mild to moderate AD and coincides with the period of anxiety and depression in AD patients when they are unaware of their surroundings and sometimes forget family members. In Chapter three, we showed that there was a significant increase in the expression of tau containing exon 10 in the amygdala of ADTDP43- cases. Consistent with this, we showed that CELF4 was increased in the amygdala (30 %) of ADTDP43- patients. Studies

investigating the regulation of tau splicing show the involvement of CELF4 as a promoter of tau exon 10 inclusion *in vitro* (Andreadis, 2005; Ladd et al., 2004; Wang et al., 2004). This increase correlates with a possible influence of CELF4 on tau containing exon 10 in the amygdala. In addition, CELF2 which promotes tau exon 2 inclusion *in vitro* (Leroy et al., 2006) was shown to be decreased in the cortical neurons of DM1 patients together with tau isoforms containing exon 2 (Sergeant et al., 2001). Hence, it is possible that the increased tau exon 10 inclusion in the amygdala of ADTDP43- cases is a consequence of the increased expression of CELF4 by promoting the recognition of exon 10 and/or recruiting other splicing factors to tau pre-mRNA during splicing regulation.

Similar to SC35 RNA, CELF1 RNA showed no significant difference in expression in all four brain regions affected in AD whereas we found a significant increase in the expression of CELF4 RNA in the amygdala of ADTDP43- cases, like SRp40 and SRp55 RNA.

The splicing activity of CELF4 was analysed by deletion analysis to be mediated through its divergent domain including its first 66 residues, in the presence of RRM1 and RRM2 or RRM2 alone (Singh et al., 2004). For example, in cTNT exon 5 alternative splicing, substitution analysis demonstrated that the divergent domain of CELF4 alone consists of 20 residues that are necessary and sufficient for cTNT exon 5 activation. This proposed that the splicing activity of CELF4 is through protein-protein interactions (Han and Cooper, 2005). Although all members of the CELF protein family are expressed in the brain, CELF4 showed an essential role in neuronal viability in the Frequent flier (*Ff*) mouse model of epilepsy (Yang et al., 2007). However, the increase in CELF4 expression was specific to the amygdala of ADTDP43- cases which suggests a special role for CELF4 within this brain region.

6.3.2.2. CELF3 is differentially expressed in the frontal cortex and amygdala of AD brain

The brain-specific CELF protein, CELF3, previously reported to be expressed in human brain at RNA (Good et al., 2000; Ladd et al., 2001; Ladd et al., 2004) and protein level (Chapple et al., 2007) showed significant increase in RNA expression in the amygdala (50 %) and frontal cortex (80 %) of AD brain independent of TDP43 inclusions in comparison to normal brain. However, at protein level, the 3B1 antibody did not show any change in expression of CELF proteins. Although the 3B1 antibody was raised against full length CELF1, it also detects other members of the CELF proteins family. We were therefore unable to identify individual CELF proteins expressed in the human brain. Hence, antibodies specific for each individual CELF protein are required to elucidate the particular expression pattern in AD brain. At this point, we cannot conclude whether this increase in CELF3 RNA results in increased CELF3 protein expression in the amygdala and frontal cortex of AD cases. In contrast, increased tau exon 10 skipping correlates with increased CELF2 in DM1 patients (Dhaenens et al., 2011). Although we cannot rule out the possibility that abnormalities unique to AD independently influence CELF3 and CELF4 expression and tau splicing pattern, we propose that the increased levels of CELF3 and CELF4 RNA may contribute to altered tau exon 10 splicing observed in the amygdala of ADTDP43- patients.

CELF proteins play a vital role in alternative splicing regulation by binding to intronic sequences whereas, in the cytoplasm, they mediate their other functions in mRNA processing (for example deadenylation, stability and translation) through their binding to 5’- and 3’-UTR and are implicated in DM with tau pathology (Gallo and Spickett, 2010). Furthermore, in DM1 patients, these functions of CELF proteins are altered. For instance, CELF1 is a splicing modulator in alternative splicing as well as being involved in translation initiation; both of which are altered in DM1 cells in accordance with the changed expression of CELF1 in DM1 cells (Savkur et al., 2004). In DM1, CELF1 protein is highly expressed in the heart and skeletal muscle (Ladd et al., 2001; Ladd et al., 2004; Timchenko et al., 1996). However, DM1 patients also show symptoms that affect the brain including impaired cognition (Wheeler, 2008). Hence, the identification of differential expression of some CELF proteins involved in tau splicing in ADTDP43- cases is consistent with the reports of altered tau splicing in AD brain.

Savkur et al. proposed a model where an increased expression of CELF1 causes insulin resistance in the skeletal muscle of DM1 patients (Savkur et al., 2001). This is due to disruption insulin receptor (IR) pre-mRNA splicing generating a non-muscle IR isoforms that lacks exon 11 (IRA) (Savkur et al., 2001). However, in brain regions affected in AD patients including the frontal cortex, temporal cortex and hippocampus, CELF1 did not show any difference in expression relative to normal controls.

Altered expression of CELF3 and CELF4 RNA may cause misregulation of their specific pre-mRNA target such as tau exon 10. This could result in increased tau exon 10 inclusion in the amygdala of ADTDP43- patients. Moreover, these CELF proteins regulate tau exon 10 splicing by binding to intronic sequences around exon 10. For instance, CELF1 mediated a switch from the active IRB isoform (inclusion of exon 11) to the production of the inactive IRA isoforms (exclusion of exon 11) in DM1 muscle cultured cells through specific binding to intronic elements in intron 10 (Savkur et al., 2001). Furthermore, it is possible that CELF3 and CELF4 mediate their splicing activity on tau exon 10 splicing through binding to intronic sequences in intron 9 or intron 10 (Chapple et al., 2007; Wang et al., 2004). It is imperative to determine precisely either through SELEX (systemic evolution of ligands by exponential enrichment) or CLIP techniques, how the brain-specific CELF3; and CELF4 control tau exon 10 splicing. These CELF proteins can modulate tau splicing in two ways; either by directly binding to intronic sequences in tau pre-mRNA and/or indirectly modulating its splicing activity through interaction with other proteins.

For instance, CELF2 binds preferably to UG and UGUU repeat motifs (Faustino and Cooper, 2005) and promotes tau exon 10 skipping when overexpressed (Dhaenens et al., 2011) while CELF6 mediates splicing activity without the presence of such consensus sequences (Gromak et al., 2003; Ladd et al., 2004). Chapple et al. reported the influence of CELF3 on tau exon 10 inclusion *in vitro* mediated through CELF3's binding to tau pre-mRNA by its RRM2 domain (Chapple et al., 2007). In accordance with this, most of the RNA-binding activity of CELF2 is credited to its RRM2 shown through mutations at the RNP motif

(Good et al., 2000). In addition, the divergent domain (between residues 312 and 335) of CELF3 is sufficient to promote tau exon 10 inclusion *in vitro*; described through deletion mutant analysis (Chapple et al., 2007). Hence, regulation of tau exon 10 splicing may be mediated through interaction with other proteins since the divergent domain modulates the splicing activity of different pre-mRNA targets.

Our results suggest a specific induction of distinct splicing factors in sporadic AD cases. The involvement of CELF3 and CELF4 in alternative splicing may be important for regulation of alternatively spliced transcripts of the *MAPT* with potential functional consequences in expression of alternative tau exon 10 and its contribution to NFT pathology in sporadic AD and other tauopathies. However, we did not obseve any clear correlation of the 4R/3R tau ratio with the increased expression of CELF proteins in the amygdale or with any pathology. Furthermore, increased activity of these CELF proteins in AD patients may be due to the increased expression of CEFL3 and CELF4 in the amygdala. We therefore propose that altered expression of splicing factors may be a contributing factor to tau pathogenesis in sporadic AD. Whether these CELF proteins are differentially expressed at the protein level, in different brain regions affected in sporadic AD is unknown. However, at RNA level CELF3 seem to be regulated in specific manner especially in the frontal cortex and amygdala where its expression was significantly increased regardless of TDP-43 inclusions.

Another level of regulation of CELF protein activity is through their phosphorylation state. Consistent with this, CELF1 is hyperphosphorylated in the tissues and cells of DM1 patients by PKC which stabilises it and causes an increase in its activity (Kuyumcu-Martinez et al., 2007).

Our data suggest that SR proteins and CELF proteins play a role in pathogenesis of AD. Other diseases that show aberrant alternative splicing mechanism include ovarian, breast and colon cancer. In a mouse model for mammary tumourigenesis, abnormal SR protein expression (increased expression of SRp40 mRNA) was observed during tumourigenesis (Stickeler et al., 1999) as well as DM1 where

CELF1 and CELF2 protein expression are decreased in the brain (Dhaenens et al., 2008; Dhaenens et al., 2011).

6.4 Correlation of splicing factors expression in AD brain and *in vitro* studies

An important question outstanding before this study was whether the splicing factors that modulate tau exon 10 inclusion *in vitro* do so *in vivo*. We showed that tau exon 10 inclusion was increased in the amygdala of ADTDP43- cases but whether this increase is due to the increase observed in the amygdala of splicing factors that modulate tau exon 10 inclusion is unknown. Statistical correlation analysis did not show a connection between the increase in expression of SRp40, SRp55 CELF3 and CELF4 RNA in ADTDP43- patients and the percentage of 4R tau increase in the amygdala of these patients (Figure 6.1, 6.2, 6.3, and 6.4). However, it is likely that the aberrant increase in tau exon 10 inclusion is due to abnormal splicing factor expression (and/or activity) in the amygdala of these AD patients and may contribute to the pathogenesis. In addition to what was reported in the literature, that CELF3, CELF4 and SRp40 promote tau exon 10 inclusion *in vitro* (Table 6.1), we show corresponding increase in the expression of these splicing factors in the amygdala of ADTDP43- patients as well as tau exon 10 inclusion in the same brain region.

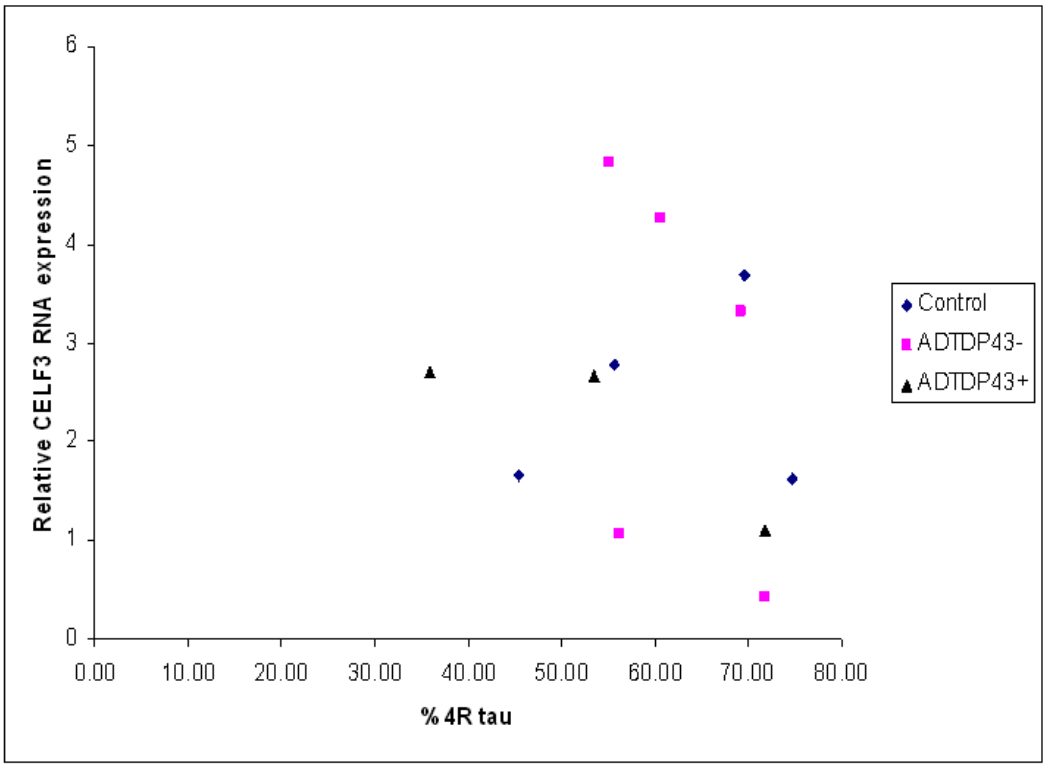

Figure 6.1. Correlation between CELF3 and percentage 4R tau in the amygdala of AD brain.

The brain specific CELF3 protein promotes tau exon 10 inclusion *in vitro* (Chapple et al., 2007); (Wang et al., 2004). We examined the correlation between relative CELF3 RNA expression and the percentage of 4R tau expressed in the same control and AD cases to determine if there is any link between them. The Pearson correlation, r in controls (r = -0.2520), ADTDP43- cases (r = 0.4290) and ADTDP43+ cases (r = 0.8821) showed no overall correlation between CELF3 and the percentage of 4R tau expressed.

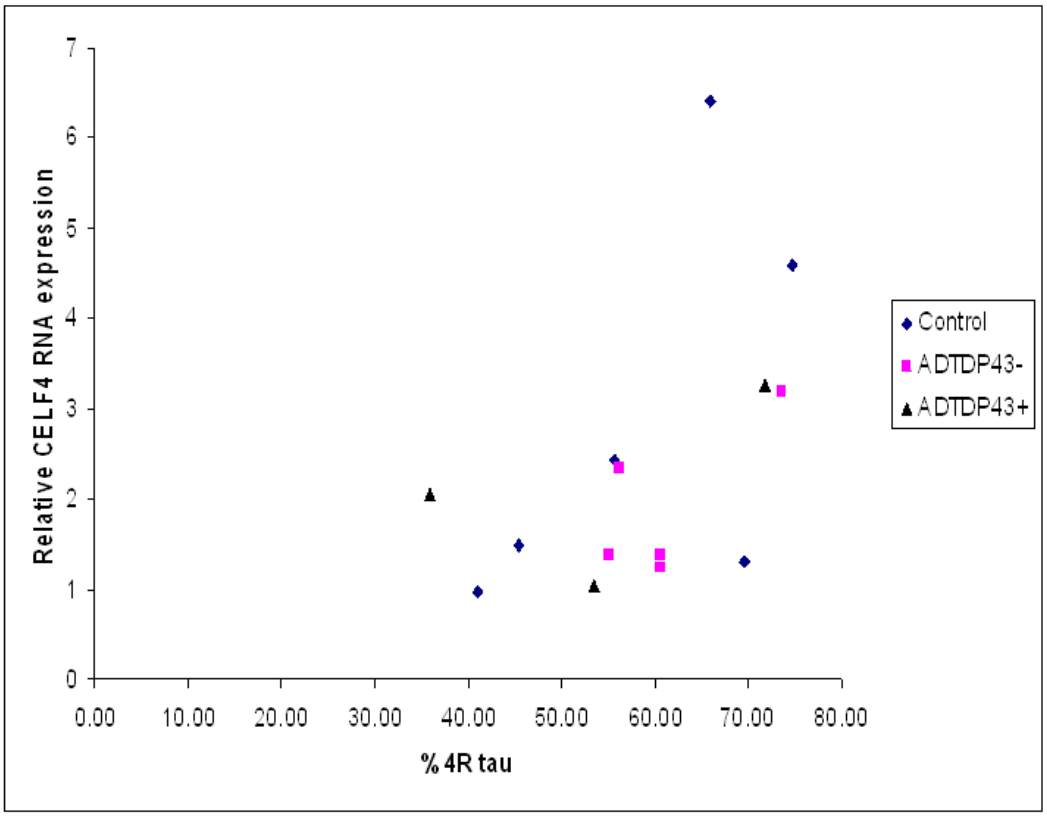

Figure 6.2. Correlation between CELF4 and percentage 4R tau in the amygdala of AD brain.

CELF4 also promotes exon 10 inclusion *in vitro* (Dhaenens et al., 2011; Wang et al., 2004). To determine if there is any link between CELF4 RNA expression and the percentage of 4R tau expressed in the same control and AD cases, we examined the correlation between them. The Pearson correlation, r in controls (r = 0.6062), ADTDP43- cases (r = 0.6852) and ADTDP43+ cases (r = 0.5533) showed no overall correlation between CELF4 and the percentage of 4R tau expressed.

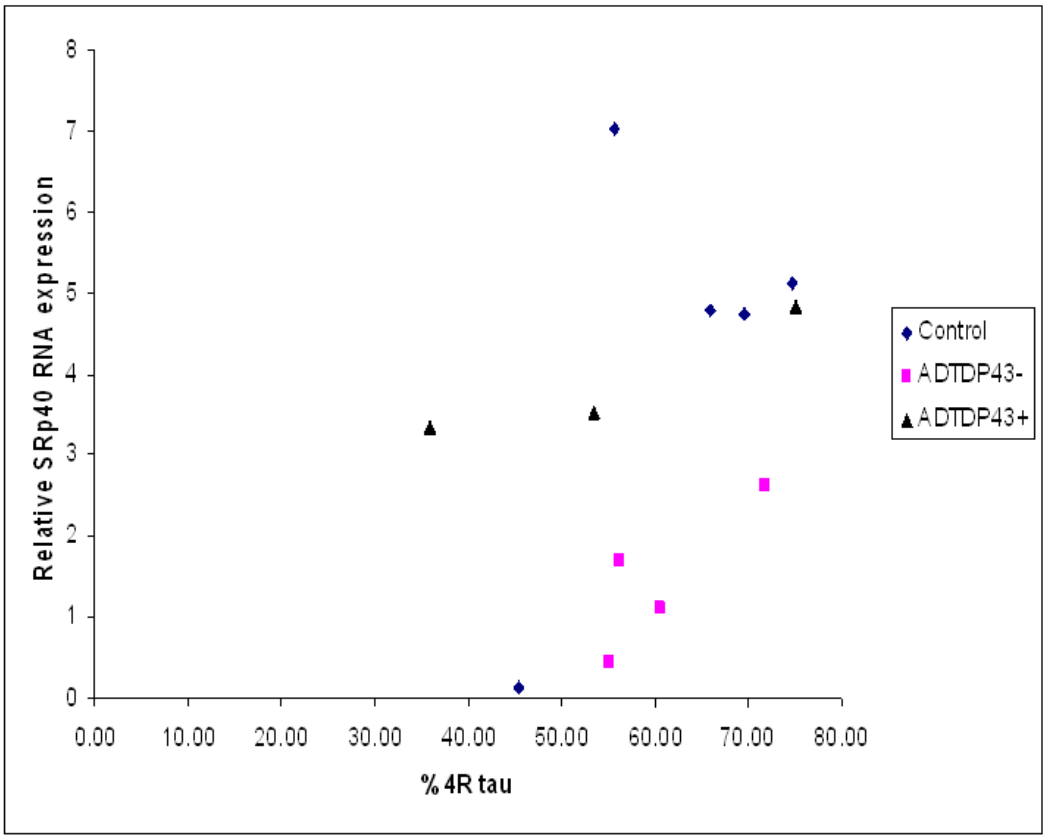

Figure 6.3. Correlation between SRp40 and percentage 4R tau in the amygdala of AD brain.

SRp40 promotes tau exon 10 inclusion *in vitro* by binding to an ESE sequence in exon 10 (Kondo et al., 2004; Wang et al., 2004). We therefore examined the correlation between relative SRp40 RNA expression and the percentage of 4R tau expressed in the same control and AD cases to determine if there is any link between them. The Pearson correlation, r in controls (r = 0.5685), ADTDP43- cases (r = 0.7716) and ADTDP43+ cases (r = 0.1106) showed no overall correlation between SRp40 and the percentage of 4R tau expressed.

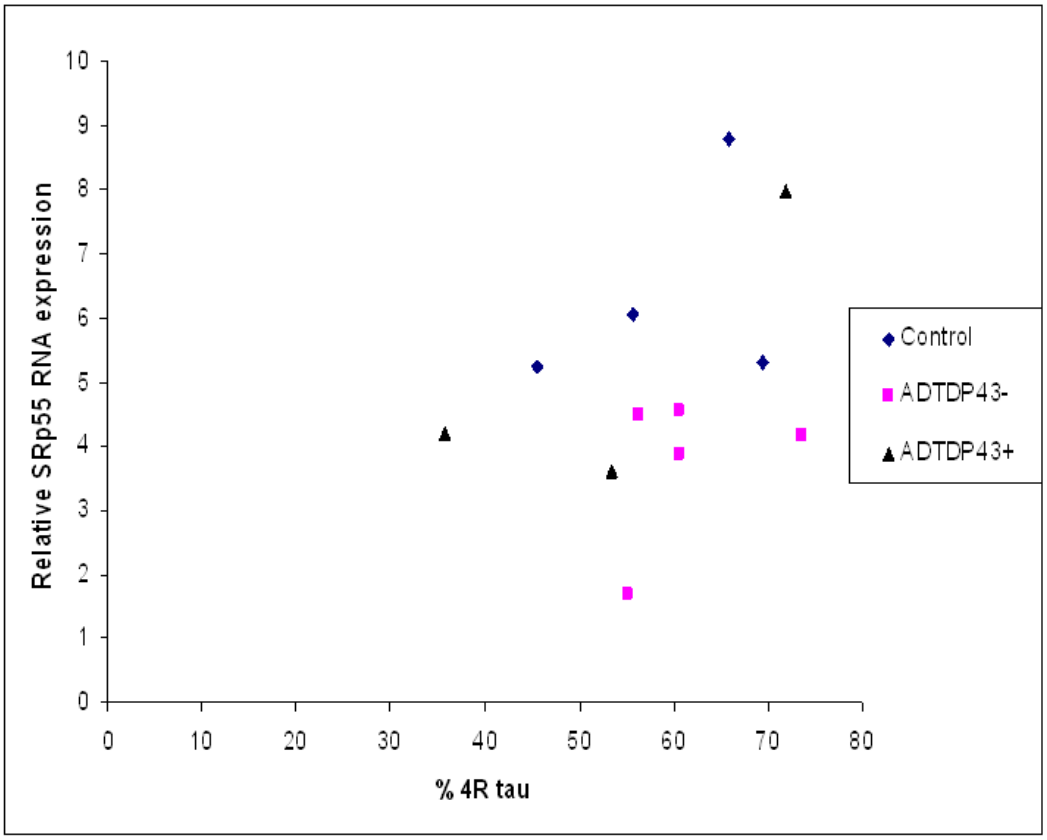

Figure 6.4. Correlation between SRp55 and percentage 4R tau in the amygdala of AD brain.

SRp55 inhibits tau exon 10 *in vitro* by binding to an ESS sequence within exon 10 (Kondo et al., 2004; Wang et al., 2004). We examined the correlation between relative SRp55 RNA expression and the percentage 4R tau expressed in the same control and AD cases to determine if there is any link between them. The Pearson correlation, r in controls (r = 0.4011), ADTDP43- cases (r = 0.3844) and ADTDP43+ cases (r = 0.8020) showed no overall correlation between SRp55 and the percentage of 4R tau expressed.

6.5 TDP-43 may modulate expression of splicing factors in AD

The identification of TDP-43 pathology in AD suggests that TDP-43 is not an exclusive marker for FTLD-U and ALS (Wilson et al., 2011). Immunohistochemical analysis was used to reveal that TDP-43 pathology co-occurred with some NFTs in AD brain, a subset of coil like structures in CBD and Pick bodies observed in PiD (Arai et al., 2006; Rohn, 2008). Approximately 30 % of AD cases have TDP-43 pathology and are associated with severe clinical symptoms including severe cognitive and functional impairment (Josephs, 2008) as well as longer disease duration (Uryu et al., 2008). TDP-43 pathology in these AD cases is limited to the limbic system including the hippocampus and amygdala as well as the temporal lobe where it is mislocalised from the nucleus to the cytoplasm as seen in all TDP-43 proteinopathies (Amador-Ortiz et al., 2007b; Hu et al., 2008; Josephs, 2008). Furthermore, immunohistochemical studies show that TDP-43 pathology is present in the hippocampus and entorhinal cortex, in a minority of normal elderly subjects (Wilson et al., 2011).

The final aim of this thesis was to determine whether TDP-43 pathology in a subset of our sample cohort (ADTDP43+) modulates the expression of CELF proteins and SR protein family members that show altered expression in AD brain without TDP-43 pathology. We found that the altered SR protein expression in ADTDP43- cases was absent in ADTDP43+ cases (Table 6.1). SRp40 and SRp55 both showed prominent increase in expression, in the amygdala of ADTDP43- cases, a region with characteristic TDP-43 pathology that also showed increased tau exon 10 inclusion. In contrast, in ADTDP43+ cases, the expression of SRp40 and SRp55 was at similar levels with controls showing no significant difference (Table 6.1). Consistent with this, the increased expression of CELF4 in the amygdala of ADTDP43- cases was absent in ADTDP43+ cases.

Table 6.1. Correlation of tau splicing factor expression and tau E10 inclusion in AD.

Splicing factors	Amygdala ADTDP43-	Amygdala ADTDP43+	Frontal cortex ADTDP43-	Frontal cortex ADTDP43+	Tau E10 *in vitro*
SRp40	↑↑↑	—	—	—	↑ (Kondo et al 2004) (Wang et al 2004)
SRp55	↑	—	—	—	↓ (Kondo et al 2004) (Wang et al 2004)
CELF3	↑	↑	↑	↑	↑ (Chapple et al.2007)
CELF4	↑	—	—	—	↑ Andreadis et al. 2005) (Wang et al. 2004)
Tau E10 inclusion	↑	—	—	—	

↑ Increased expression ↓ Decreased expression — No change

A possible explanation for this is that TDP-43 may be protective of the increase in tau exon 10 inclusion through its suppression of SR proteins that modulate tau exon 10 splicing probably through sequestration the splicing factors (that promote tau exon 10 inclusion) in its nuclear cluster (for example SRp55). However, the presence of TDP-43 inclusions is correlated with sever clinical symptoms in ADTDP43+ cases (Josephs et al., 2008). Interestingly, in addition to the increased expression of CELF3 in ADTDP43- cases both in the amygdala and frontal cortex, we found a slightly more pronounced increased in CELF3 expression in ADTDP43+ cases in both brain regions (Table 6.1). It is yet to be determined whether there are any interactions between these splicing factors and TDP-43 except for SRp55 which was found in a nuclear cluster of TDP-43 interacting proteins (Freibaum et al., 2010).

Recently, Polymenidou et al. reported that TDP-43 modulated the expression levels of gene transcripts implicated in ALS using adult mouse brain (Polymenidou et al., 2011). They demonstrated by CLIP that TDP-43 protein directly binds to *FUS/TLS* mRNA (at the 3'UTR as well as intron 6 and 7) which encodes for FUS/TLS mutations that result in ALS. RT-qPCR showed that *FUS/TLS* mRNA and protein levels were significantly decreased by roughly 40 % of their normal levels after TDP-43 depletion (Polymenidou et al., 2011). In contrast, TDP-43 protein binding to *GRN* mRNA (encodes for progranulin mutations in FTLD) was significantly increased by up to six-fold compared to controls (Polymenidou et al., 2011).

Another mRNA associated with TDP-43 is the histone deacetylase 6 (HDAC6) mRNA, whose expression was decreased in the absence of TDP-43 using antisense oligonucleotides in mouse brain (Polymenidou et al., 2011). HDAC6 is crucial for protein aggregate degradation (Fiesel et al., 2009). HDAC6 has two deacetylase domains; a dyenin motor protein domain which transports cargo to the autophagic pathway for degradation; and a zinc finger domain that binds to polyubiquitin (Kawaguchi et al., 2003). Hence, HDAC6 through its binding to poly-ubiquitinated misfolded proteins as well as the dynein motor protein may be transported to an aggresome for degradation (Kawaguchi et al., 2003). In neurodegeneration, pathological aggregates consist of misfolded proteins such as

Aβ, tau and TDP-43. Interestingly, although there are several binding sites for TDP-43 in tau pre-mRNA there was no significant difference in tau splicing pattern or levels of tau mRNA when TDP-43 was depleted in mouse brain (Polymenidou et al., 2011).

Buratti et al. demonstrated that TDP-43 distinctively binds to a (UG)n element near the 3' splice site of CFTR exon 9 to promote its skipping (Buratti and Baralle, 2001). In contrast, TDP-43 activates SMN exon 7 inclusion, which contributes to SMA pathogenesis (Bose et al., 2008). There are a group of splicing factor families such as hnRNP and SR proteins that can either promote or repress inclusion of alternative exons (Douglas and Wood, 2011). In addition, members of the CELF protein family bind to UG or UGUU intronic elements and mediate their splicing activity as either repressors or activators of splicing (Chapple et al., 2007; Gromak et al., 2003). We therefore propose that TDP-43 promotes or represses splicing activity depending on the target or its influence on a splicing regulator. For instance, TDP-43 can influence tau exon 10 splicing indirectly by binding to another splicing factor such as hnRNP or to an element upstream or downstream of exon 10. Indeed, the C-terminus of TDP-43 interacts with members of hnRNP protein family, crucial for the formation of an hnRNP complex essential for CFTR exon 9 skipping (Buratti et al., 2005).

6.6 Future work

6.6.1 Expression levels of splicing factor pre-mRNA targets in AD

An important question highlighted from this study is whether the altered expression of SR proteins and CELF proteins affects other pre-mRNA targets also regulated by the specific members of splicing factor families that show altered expression in AD patients. This will elucidate whether the aberrant activity of CELF and SR proteins in AD results in altered splicing pattern of their other pre-mRNA targets. For instance in the temporal cortex of AD patients with aberrant tau exon 10 splicing, tra2B1 (htra2β), the SR-related protein which promotes tau exon 10 inclusion (Jiang et al., 2003; Kondo et al., 2004) showed alteration in its splicing pattern (Glatz et al., 2006).

It is also crucial to determine if the altered expression and/or activity of the SRp40, SRp55, CELF3 and CELF4 shown in this study is disrupted in other brain regions affected in AD patients such as the transentorhinal and entorhinal cortex of AD brain from which tau pathology begins (Braak and Braak, 1992). Because AD arise from the transentorhinal cortex we need to compare the splicing pattern of tau exon 10 and expression of splicing factors in AD brain with that of normal age-matched controls in order to confirm our findings. Also, the role specific SR proteins play during tau alternative splicing *in vivo* should be investigated. In addition, it is likely that different human brain regions express different subsets of splicing factors. However, to study the individual SR proteins and CELF proteins, it is vital to develop an antibody to recognise the specific splicing factors.

6.6.2 Expression levels of SR proteins and CELF proteins in other sporadic tauopathies.

Aberrant tau exon 10 splicing has been reported for other tauopathies including CBD, PSP and PiD. In CBD and PSP tauopathies, there is increased expression of tau containing exon 10 transcripts in the brain. We proposed from this study that aberrant tau exon 10 splicing due to altered expression and activity of splicing

factors can contribute to AD pathogenesis. Whether these SR proteins and CELF proteins expression is altered in these other tauopathies is unknown. It would be interesting to determine whether these SR proteins (SRp40) and CELF proteins (CELF3 and CELF4) that regulate tau exon 10 splicing have increased expression in PSP and CBD. In PiD, there is an increase in the expression of 3R tau, however, we do not know whether the expression of CELF proteins and SR proteins promoting tau exon 10 splicing are decreased in this tauopathy. Similar to how we determined the expression levels of SR proteins and CELF protein transcripts in AD brain, we can use RT-qPCR to determine the expression levels of the specific splicing factors in the brain compared to normal control individuals.

6.6.3 Identification of phosphorylation state of SR proteins and CELF proteins in AD brain compared to normal human brain

Analysis of the phosphorylation state of the different splicing factors in AD brain can be attempted by mass spectrometry. Immunoprecipitation of specific splicing factors can be enriched from human brain using specific SR protein antibody prior to mass spectrometry analysis. For example, protein bands corresponding to AD-SRp40 (and control-SRp40) from corresponding brain can be excised from Coomassie blue-stained gels and subject to mass spectrometric analysis. The excised bands will be reduced, alkylated and digested with trypsin or Asp-N and the resultant peptides separated by reversed-phase chromatography.

Mass spectral data can then be searched against a database containing all isoforms of human SRp40 using the Mascot searching algorithm (Matrix Science). This will determine the phosphorylation state of SR proteins which should have higher masses compared to their normal molecular weight. A similar analysis was reported for PHF-tau form AD brain (Hanger et al., 2007). This would elucidate whether specific splicing factors have aberrant phosphorylation in AD brain.

6.6.4 Splicing factors in tauopathies

This study used gene expression analysis to elucidate the possible level of activity of splicing factors/RNA-binding proteins in sporadic AD. The importance of accurate alternative splicing of tau during RNA metabolism is well established and our results implicate specific members of CELF proteins and SR proteins in brain regions affected in AD, particularly, the amygdala and frontal cortex (Table 6.1). For the first time, we show that aberrant expression of splicing factors in AD brain regions may play a role during pathogenesis. We observed an increase in tau exon 10 containing transcripts in the amygdala and hippocampus of AD patients and consistent with this, found that specific CELF and SR proteins are up-regulated in these brain regions (Figure 6.5). Given the ability of SR proteins and CELF proteins to influence RNA processing events, our results suggest that a number of pre-mRNAs may undergo changes in alternative splicing in different brain regions of AD pathogenesis. Together with previously performed functional analysis of SR proteins and CELF proteins in tau splicing *in vitro*, these findings implicate that the altered expression profile of SRp40, SRp55, CELF3 and CELF4 might be responsible for changes in tau alternative splicing in sporadic AD (Figure 6.5). These splicing factors can thus be therapeutic targets to correct abnormal tau exon 10 splicing. Future investigation into the relationship among specific members of the CELF and SR protein families and their roles during tau splicing *in vivo* is essential. Also, the level of activity of these CELF and SR protein families in different brain regions especially in AD brain will provide other avenues for therapeutic intervention in AD and other sporadic tauopathies.

Figure 6.5. SRp40, SRp55, CELF3 and CELF4 misregulation may cause tau pathology in AD brain.

Members of SR protein and CELF protein families; SRp40, SRp55, CELF3 and CELF4 are modulators of tau exon 10 inclusion *in vitro*. Their altered expression in brain regions affected in AD may lead to an increase in tau exon 10 inclusion due to increased activity. Hence, misregulation of target pre-mRNA including tau causing mis-regulation of tau transcripts and contribute to NFT pathology with subsequent destabilisation of functional tau to stabilise microtubules.

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Appendix

1.1

Pathological information for AD cases

Case	Pathology	
A249/07 Braak stage VI with amyloid angiopathy	FC	This region showed numerous neuropil threads, many neurofibrillary tangles and frequent neuritic plaques. Also, there were numerous amyloid plaques.
	TC	The middle temporal gyrus shows moderate neuronal loss with superficial spongiosus. Frequent neuritic plaques are demonstrated by Tau and Bielschowsky in the middle temporal gyrus. TDP-43 shows no specific MND-type inclusions.
	A	The amygdala showed severely affected by neuronal loss.
	H	The hippocampus shows of pyramidal neurones with many ghost tangles in CA1 segment, amounting to hippocampal sclerosis. Tau shows numerous neuropil threads and many neurofibrillary tangles in the hippocampus
	C	No pathological information available
A205/07 Braak stage VI with amyloid angiopathy and additional TDP-43 positive	FC	Sections of the frontal lobe show large numbers of neuritic plaques, neurofibrillary tangles and neuropil threads. The Aβ immunohistochemistry reveals extensive deposition mainly in the form of cored and diffuse plaques. TDP-43 does reveal very occasional neuronal inclusions within the frontal neocortex
	TC	There is extensive tau deposition in the form of tangles and plaques and neuropil threads within the cortex. The Aβ again shows extensive deposition in the form of cored and diffuse plaques and amyloid angiopathy.
	A	Sections from the amygdala show large numbers of tangles and neuropil threads in tau immunohistochemistry.Aβ immunohistochemistry showed large numbers of plaques within the amygdala. The amygdala also contains very mild, occasional neuropil threads. TDP-43 inclusions predominantly neuronal cytoplasmic inclusions.
	H	Presence of large numbers of neuritic plaques in the hippocampus associated with neuropil threads and neurofibrillary tangles. Also, extensive Aβ deposition in the form of cored and diffuse plaques. A number of cytoplasmic TDP-43 inclusions are

		also persent.
	C	Tau is negative. The Aβ does reveal some plaques mainly in the molecular layer. There are occasional inclusions within the molecular layer, which appear mainly to be neuropil thread-like structures as well as occasional dystrophic neurites extending from the Purkinje cell layer.
A349/08 Braak stage VI with amyloid angiopathy and limbic TDP43 pathology	FC	The tau immunohistochemistry in this region showed high density of neuropil threads with neurofibrillary tangles and dystrophic neurites and scattered neuritic plaques. The Aβ, showed a high density of plaques which were predominantly diffuse. Also, a number of cored plaques were seen within the cortex.
	TC	The temporal neocortex showed moderate density of neuropil threads and moderate numbers of neurofibrillary tangles with mild to moderate numbers of plaques in the middle temporal gyrus. The superior temporal gyrus contains occasional neuropil threads and tangles.
	A	Sections from the amygdala showed scattered neuritic plaques. Tau immunohistochemistry highlights large numbers of neurofibrillary tangles and a high density of neuropil threads and scattered neuritic plaques. The TDP43 reveals positivity in a number of neurones in the form of neuronal cytoplasmic inclusions, some being tangle-like, and some being more crescent-like. There are a number of neuropil threads identified also.
	H	Marked neuronal loss. Large numbers of neurofibrillary tangles and high density of neuropil threads. Moderate to large numbers of neuritic plaques. The Aβ reveals large numbers of cored and diffuse plaques in the hippocampus
	C	No pathology
A348/07 Braak IV with focal amyloid angiopathy	FC	Sections from the frontal lobe reveal no neuritic plaques but very occasional neurofibrillary tangles The TDP-43 is negative. The Aβ reveals a number of cored and diffuse plaques in the cortex.
	TC	Sections from the temporal neocortex show only very occasional neurofibrillary tangles and No neuritic plaques are seen. The Aβ reveals deposition in the form of cored and diffuse plaques within the cortex.
	A	Sections from the amygdala do show large numbers of neuritic plaques and neurofibrillary tangles and

		neuropil threads and occasional grain-like structures.
	H	Tau immunohistochemistry reveals a number of neurofibrillary tangles within the pyramidal cell layer. There is a moderate density of neuropil threads. The TDP-43 reveals very occasional cytoplasmic neuronal inclusions and the neurites.
	C	TDP-43 and tau are negative. Sections from the cerebellum show good preservation of the Purkinje cell layer.
A076/09 Braak V, CERAD definite; TDP-43 pathology (predominantly in amygdala & hippocampus)	FC	Tau reveals many tangles, threads, and dystrophic neurites. A4 for β-amyloid shows severe cortical capillary amyloid angiopathy; high numbers of diffuse plaques and up to frequent neuritic plaques.
	TC	Occasional cytoplasmic TDP-43 inclusions are noted in the cortical neurones.
	A	There is significant neuronal loss in the amygdala. Tau reveals high density of neuropil threads, tangles and also scattered neuritic plaques There are numerous TDP-43 positive neuronal cytoplasmic inclusions in the amygdala and adjacent limbic areas.
	H	There is severe tau pathology with high density of neuropil threads, dystrophic neurites and tangles. TDP-43 few inclusions are noted
	C	No significant pathologies are noted.
A037/04 Braak stage IV	FC	There are frequent neuritic plaques in the cortex and large amounts of A4 protein deposits. Only a few neurofibrillary tangles are seen.
	TC	There are frequent neuritic plaques and neuropil threads and a large number of primitive plaques in the cortex. A few tangles are also seen.
	A	No pathological information available
	H	There are a few neuritic plaques and many neurofibrillary tangles in the pyramidal cell layer. Granulo-vascular degeneration and neuropil threads are also present.
	C	There is mild to moderate loss of Purkinje cells and increased cellularity of the molecular layer.
A039/02 Braak stage IV	FC	The frontal cortex shows some superficial spongiform change. There are large numbers of neuritic plaques and neurofibrillary tangles present.
	TC	The temporal neocortex contains large numbers of neurofibrillary tangles and there are also focally large numbers of neuritic plaques.
	A	No pathological information available
	H	Sections from the hippocampus show neuronal loss,

		most notably in the pyramidal cell layer, CA1. There are very large numbers of neurofibrillary tangles present within the hippocampus. There are moderate numbers of neuritic plaques and large numbers of dystrophic neurites present.
	C	Sections from the cerebellum show a well-preserved Purkinje cell layer. No abnormality is seen.
A331/07 Braak stage V with mild amyloid angiopathy.	FC	This region showed numerous neuropil threads and neurofibrillary tangles and neuritic plaques as well as numerous amyloid plaques.
	TC	The neuronal loss in this region was mild in the middle temporal gyrus and showed moderate neuritic plaques.
	A	The amygdala showed prominent neuronal loss with spongiosus.
	H	The hippocampus also showed prominent neuronal loss with spongiosus and numerous amyloid plaques
	C	No pathological information available
A098/04 Braak stage VI with mild amyloid angiopathy	FC	There is mild neuronal loss in the cortex. Tau demonstrates moderate to numerous neurofibrillary tangles and neuropil threads with moderate numbers of neuritic plaques.
	TC	numerous amyloid plaques are present, both diffuse and mature types, in the rest of the temporal cortex. There are numerous neurofibrillary tangles and neuropil threads with moderate to frequent neuritic plaques.
	A	No pathological information available
	H	There is mild to moderate neuronal loss in the hippocampus with some ghost tangles. Also, there were many amyloid plaques in the hippocampus.
	C	No pathological information available
A191/07 Braak stage VI. No amyloid angiopathy was identified.	FC	There are large numbers of neuritic plaques and neurofibrillary tangles. There was extensive deposition of cored and diffused plaques.
	TC	The temporal neocortex reveals large numbers of neuritic plaques and neurofibrillary tangles There is also a high density of neuropil threads within the cortex of these regions. The Aβ reveals extensive deposition of the protein in the form of cored and diffuse plaques. There is no evidence of amyloid angiopathy.
	A	Sections from the amygdala reveal large numbers of neurofibrillary tangles and a high density of neuropil threads identified. Large numbers of

		neuritic plaques are also seen. The Aβ reveals extensive deposition mainly in the form of diffuse plaques but also a number of cored plaques are present.
	H	There are moderate numbers of neurofibrillary tangles and neuritic plaques within the hippocampus. Also there were large numbers of neuropil threads and extensive deposition of the protein in the form of cored plaques and diffuse plaques.
	C	No pathological information available
A192/07 Braak stage V with severe amyloid angiopathy.	FC	In the middle frontal cortex there is severe neuronal loss with superficial spongiosis. Numerous tau positive tangles and threads are noted as well as high density of plaques many of which were 'mature' neuritic. Diffuse plaques were also numerous.
	TC	There is severe amyloid angiopathy in the temporal lobe sections. There are distended perivascular spaces around parenchymalblood vessels, predominantly in the white matter. There is patchy pallor of the white matter. TDP-43 reveals diffuse cytoplasmic staining in neurons.
	A	In the amygdala severe pathology is seen in forms of numerous threads, tangles and plaques.
	H	The hippocampus is atrophic with severe neuronal loss in the CA1, CA3 and 4 segments. Tangles, ghost tangles were numerous and few plaques were seen.
	C	No pathological information available
A210/05 Braak stage V with moderate amyloid angiopathy	FC	Neurofibrillary tangles were numerous both in the superficial and deeper cortical layers. Neuropil threads and tau-positive dystrophic neurites contributing to plaque formation were also frequent.
	TC	Moderate neuronal loss with superficial spongiosis. Also, there were a high number of plaques and tangles.
	A	The amygdala contained numerous tau-positive tangles as well as moderate amyloid angiopathy.
	H	In the hippocampus, there were numerous tangles and moderate numbers plaques in the posterior part of the hippocampus.
	C	Mild, patchy Purkinje cell loss is noted. The dentate nucleus is well populated by neurones. Tau is immuno-negative.
A240/06 Braak stage V with mild amyloid angiopathy	FC	Sections from the frontal lobe showed scattered moderate numbers of neuritic plaques and occasional neurofibrillary tangles and neuropil threads. Extensive deposition of diffuse and cored

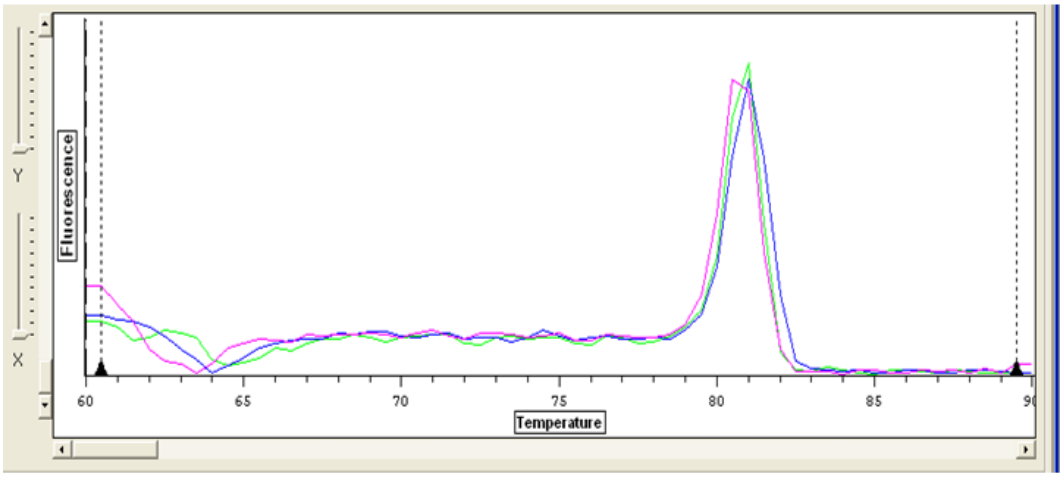
		plaques.
	TC	The temporal neocortex showed some superficial spongiform change. There were scattered neuritic plaques and large numbers of neurofibrillary tangles and neuropil threads. Extensive deposition of amyloid plaques and diffuse plaques.
	A	Tau-positive tangleswere evident.
	H	Sections from the hippocampus showed evidence of loss of neurons within the pyramidal cell layer. Within the hippocampus there were large numbers of neurofibrillary tangles and neuropil threads as well as occasional neuritic plaques. Moderate deposition of amyloid plaques within the hippocampus.
	C	No pathological information available
A141/07 Braak stage VI with amyloid angiopathy	FC	There was moderate neuronal loss as well as high numbers of tau positive tangles and threads. Also, there were numerous and many amyloid plaques.
	TC	Severe neuronal loss was seen, with superficial spongiosis. Numerous neurofibrillary tangles and neuropil threads as well as high numbers of plaques many of them were neuritic.
	A	Severe pathology was seen in the amygdala in forms of numerous threads, tangles and plaques.
	H	Tangles, ghost tangles were numerous with occasional plaques.
	C	No pathological information available
A050/04 Braak stage V	FC	There were a few plaques in the cortex but a large number of neurofibrillary tangles and neuropil threads were seen.
	TC	There were frequent neuritic plaques, tangles and neuropil threads as well as evidence of amyloid angiopathy. Also, loss of neurones.
	A	No pathological information available
	H	There is loss of neurones in CA1 region and subiculum. There are many tangles and plaques in all layers of the hippocampus
	C	No pathological information available
A058/07 Braak stage VI	FC	Sections from the frontal lobe reveal large numbers of neuritic plaques and neurofibrillary tangles within the cortex. Confirmed large numbers of cored and diffuse plaques and amyloid angiopathy, both within the cortical vessels and leptomeningeal vessels. The amyloid angiopathy was very extensive.
	TC	Large numbers of neuritic plaques and neurofibrillary tangles were observed. There was also a high density of neuropil threads within the cortex. There was extensive deposition of the Aβ

		within the cortex in the form of cored and diffuse plaques. There was also amyloid angiopathy
	A	The sections of the amygdala show focally large numbers of neuritic plaques and neurofibrillary tangles and a very dense collection of neuropil threads.
	H	Sections from the hippocampus reveal some mild loss of neurons in the pyramidal cell layer. There were large numbers of neuritic plaques and neurofibrillary tangles, very high density of neuropil threads/dystrophic neurites. Also, there were large numbers of cored and diffuse plaques and evidence of amyloid angiopathy.
	C	Sections from the cerebellum showed good preservation of the Purkinje cell layer. No other abnormality was seen.
A160/06 Braak stage VI	FC	Moderate numbers of neuritic plaques and numerous neurofibrillary tangles and neuropil threads were observed.
	TC	There were numerous tau-positive neurofibrillary tangles and neuropil threads and numerous amyloid plaques.
	A	In the amygdala, there was mild neuronal loss with moderate neurofibrillary tangles, frequent neuritic plaques and neuropil threads.
	H	The hippocampus showed prominent loss of pyramidal neurones, with many ghost tangles. Also, numerous neurofibrillary tangles and frequent senile plaques were demonstrated. A4 demonstrates numerous amyloid plaques and mild amyloid angiopathy.
	C	There was mild Purkinje cell loss
A187/07 Braak stage V with mild amyloid angiopathy	FC	Moderate neuropil threads, sparse neurofibrillary tangles and neuritic plaques were observed. numerous amyloid plaques, mainly of diffuse type,
	TC	Moderate to frequent neuritic plaques were noted
	A	Numerous neuropil threads and many neurofibrillary tangles were observed.
	H	The hippocampus showed mild loss of pyramidal cells, numerous neuropil threads and many neurofibrillary tangles as well as numerous amyloid plaques. Also, numerous diffuse and moderate cored plaques were observed.
	C	There was an old cystic infarct in the cerebellar cortex.
A122/04 Braak stage V with moderate	FC	Moderate numbers of neurofibrillary tangles and neuropil threads with only occasional neuritic plaques were observed. There was also some Tau

amyloid angiopathy		positive astrocytes in places.
	TC	The temporal cortex showed moderate amyloid angiopathy, numerous tau-positive neurofibrillary tangles and neuropil threads.
	A	Neurofibrillary tangles and neuropil threads were moderately frequent.
	H	There was moderate loss of pyramidal neurons in the hippocampus as well as neurofibrillary tangles and numerous amyloid plaques, mainly of diffuse type.
	C	There was mild Purkinje cell loss.
A065/04 Braak stage VI without any associated amyloid angiopathy	FC	Numerous neurofibrillary tangles, including some ghost tangles and neurophil threads, were seen in the frontal cortex.
	TC	There were numerous amyloid plaques, both diffuse and mature type; numerous neurofibrillary tangles and neurophil threads and also frequent neuritic plaques in temporal lobe areas.
	A	The amygdala showed frequent neurofibrillary tangles.
	H	There was mild to moderate loss of pyramidal neurons of the hippocampus. Neurofibrillary and ghost tangles and some amyloid plaques were obvious.
	C	No pathological information available

1.2. Melting curve verification for primers

CELF1



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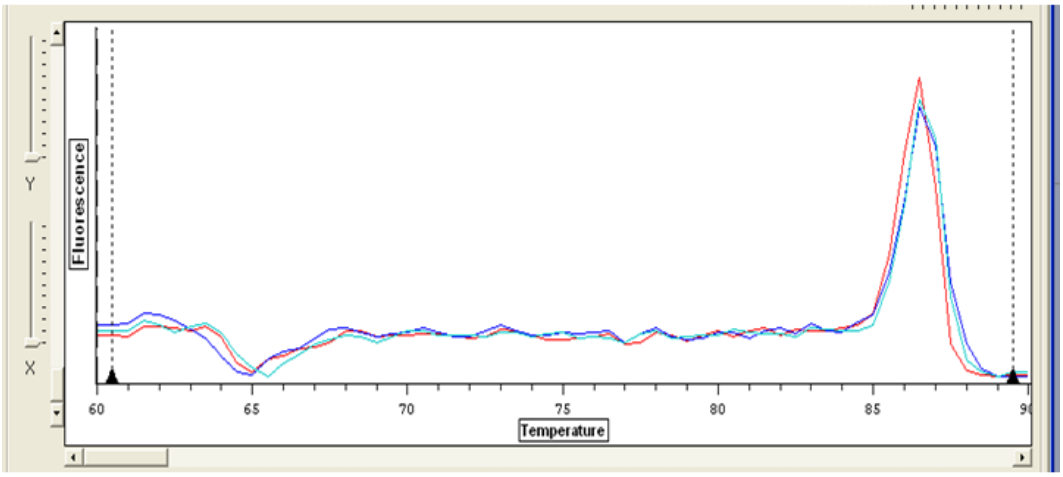

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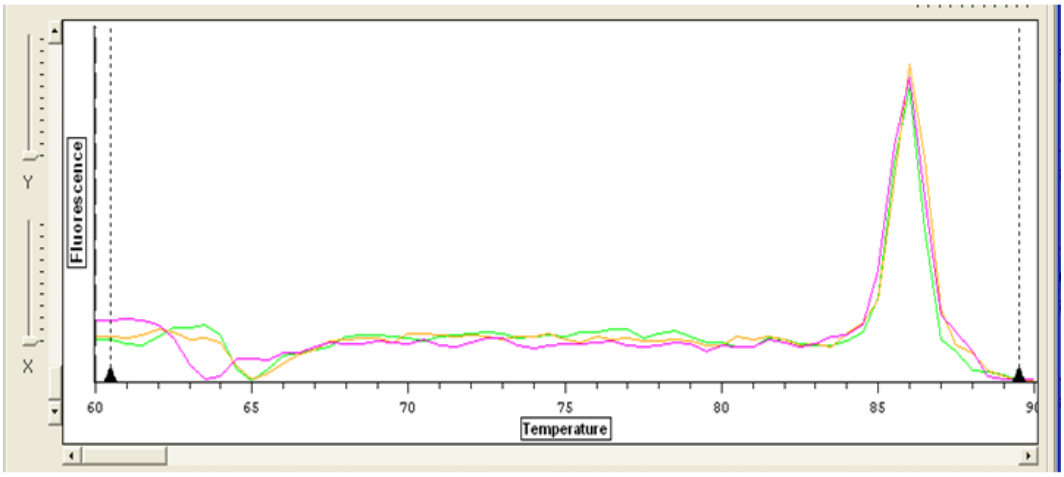

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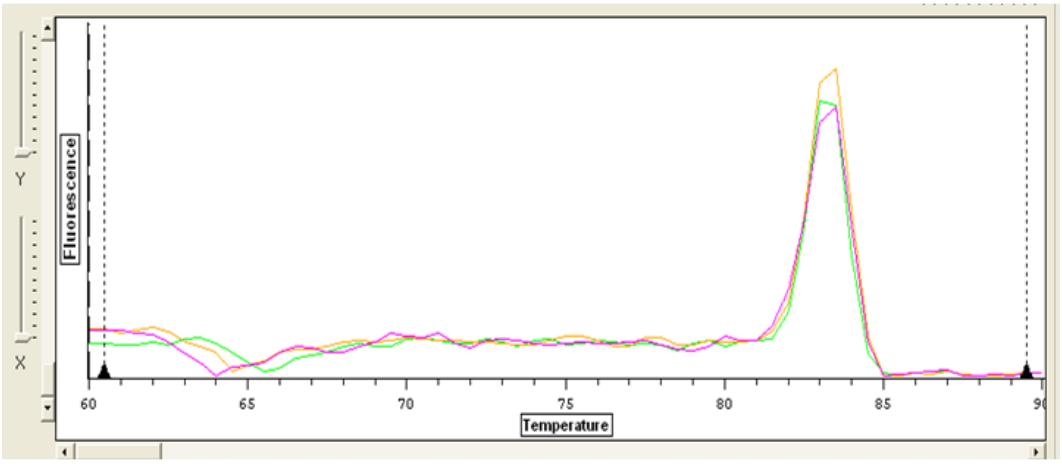

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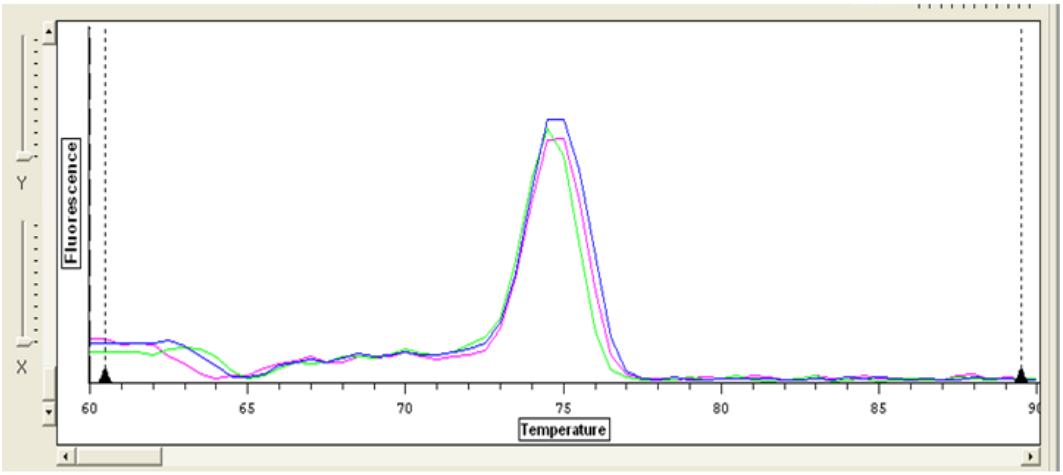

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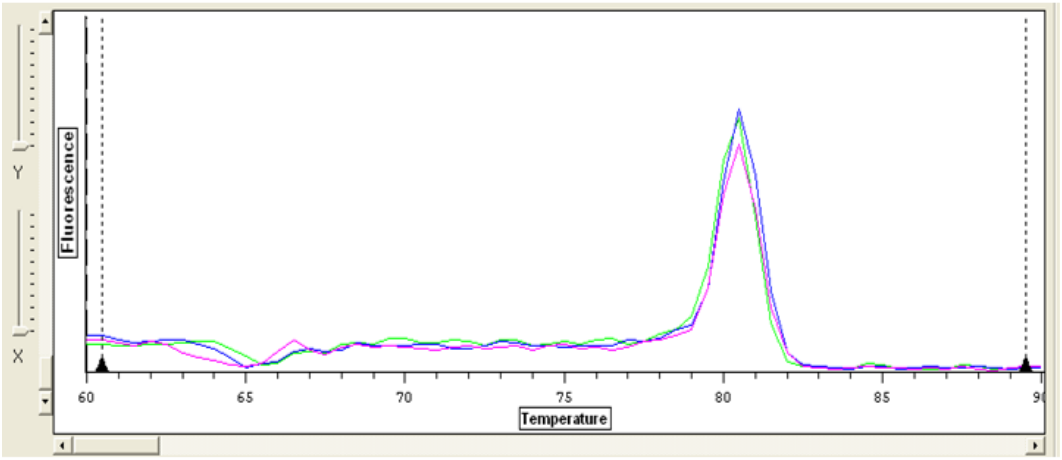
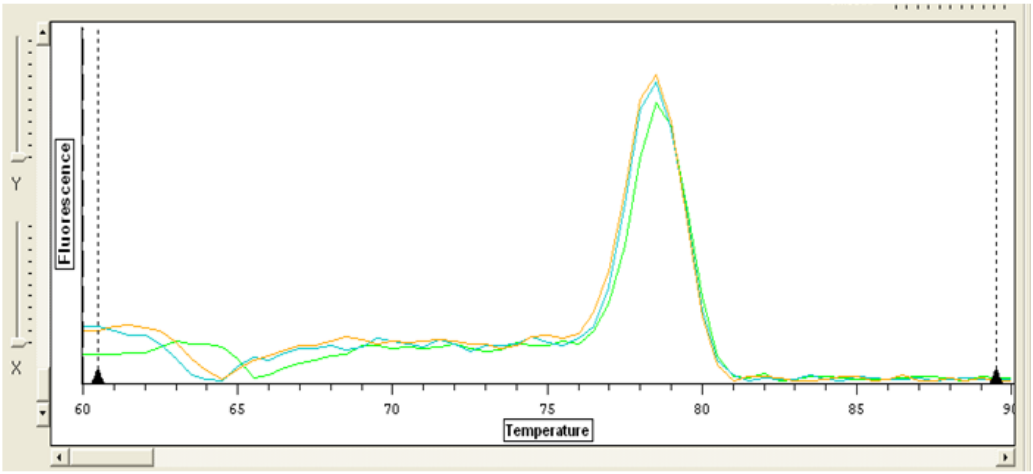


Beta-actin



GAPDH